PRODUCTION OF EXTRACELLULAR ENZYMES BY *TRICHODERMA* SPECIES AND THEIR USE FOR PROTOPLAST FORMATION IN *VOLVARIELLA VOLVACEA*

By

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A Thesis

Summitted in Partial Fulfilment

of the Requirement for the Degree of

Master of Philosophy

June, 1984

Division of Biology

Graduate School

The Chinese University of Hong Kong

This thesis is dedicated

to Lord, my God.

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ACKNOWLEDGEMENTS

First of all, I wish to express my sincere thanks and deepest gratitude to Dr. Yun-Shen Bau, my supervisor, for his constant guidance and invaluable discussions in my graduate study.

I am deeply grateful to Prof. S. T. Chang, Dr. K. Y. Chan and Dr. J. F. Peberdy for their willingness to serve on my thesis committee. My grateful thanks are also due to Dr. S. F. Li for her suggestions and discussion for this study.

I would like to thank Mr. C. C. Wai for his assistance in many ways. It is also my pleasure to express my appreciation to Mr. K. S. Au, Mr. W. C. Lau, Ms. S. W. Chiu and Ms. M. L. Yu for their advices and discussions during my graduate study.

Finally, my thanks are due to my brothers, Conred and Daniel, for their help during the publication of this thesis.

ABSTRACT

Experiments were carried out to find a suitable condition for enzyme production in several Trichoderma species (two strains of T. harzianum, a strain of T. koningii, a strain of T. longibrachiatum and a local isolated strain, TX). Volvariella volvacea hyphal wall, chitin and Laminaria meal were used as inducers. Enzyme activities of α-, β-glucanases and chitinase were detected.

When Laminaria meal was added to medium, higher level of α-glucanase was found as compared to other inducer combinations. However, the enzyme yield was low as compared to the yields of β-glucanase and chitinase. When the fungi were grown in basic TLE medium without inducers added, only small amount of α-glucanase could be found in Day 4 culture. In the same medium condition, Day 6 to Day 8 cultures generally allowed for maximum enzyme accumulation in the culture filtrate. However, a high level of β-glucanase and chitinase in Day 4 culture in basic TLE medium by some Trichoderma strains were detected. As comparing the effect of growth period, the above mentioned enzymes reached their maximum yield in Day 4 culture, earlier than that of α-glucanase. Hyphal wall of Volvariella volvacea V5

was effective on inducing β-glucanase and chitinase production. However, different Trichoderma species had various responses to the inducers.

Enzyme samples were collected from different Trichoderma strains under various inducer combinations, and were tested their abilities in releasing protoplast from two strains of Volvariella volvacea, V4 and V5, and an edible mushroom, Pleurotus sajor-caju PL27. It was found that protoplasts from V5 burstéd rapidly as soon as they were released from hyphae. However, protoplasts from V4 were stable. High yield of protoplasts could be prepared from PL27. The best enzyme sample for protoplast release from V4 and PL27 was from Day 4 culture of T6 (Trichoderma longibrachiatum) grown in medium supplemented with V5 hyphal wall and chitin. It appeared that β-glucanase and chitinase were essential components for PL27 protoplast formation. Amount of α-glucanase in enzyme samples seemed to be unrelated to protoplast yield from PL27, but did in V4. Thus, small amount of V4 protoplasts was associated with low level of α-glucanase in enzyme samples.

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1 INTRODUCTION

Recently, an increasing number of research work has been done in using protoplast obtained from mycelial cells for its physiological, biochemical and genetic studies. For example, protoplasts from filamentous fungi have been widely used in studies on biosynthesis and function of the cell wall. Moreover, protoplast fusion is a potential method for strain improvement in manipulating hybrid between closely related as well as unrelated fungi. Volvariella volvacea, a well known delicacy and extensively cultivated mushroom, presents a rigid cell wall which is probably a restrictive barrier in the establishment of heterokaryons (Santiago, 1982a). Although protoplast formation is of current interest because of its possibilities in pure and applied genetics, very few reports are concerned with Volvariella volvacea (Hamlyn et al., 1981; Qiu et al., 1982; Santiago, 1982a). The most common methods of protoplast preparation in laboratory practice are the enzymatic methods (Gibson and Peberdy, 1972; Hashiba and Yamada, 1982; Santiago, 1982a). Enzyme preparations from several Trichoderma species have been used. One of the richest sources of

lytic enzymes for filamentous fungi walls is the enzymes mixture of Trichoderma harzianum (Santiago, 1982b). In Qiu et al. study (1982), Trichoderma longibrachiatum was useful. Accordingly, it is of considerable interest to obtain preparation from different Trichoderma species which has a chance of obtaining different, though perhaps partially duplicating, enzyme components. The lytic enzyme preparation has be found to have α-, β-glucanases and chitinase activities (Hasegawa and Nordin, 1969; de Vries and Wessels, 1972). Under some conditions, certain substances can induce more enzymes production in Trichoderma.

The present work is in the study of enzyme inducing powers of some substances. It is also hoped to select a Trichoderma species which is able to produce more effective lytic enzyme with suitable inducers added. Volvariella volvacea hyphal wall, chitin and Laminaria meal are used as inducers.

This thesis includes two parts. The first part is carried out to examine the inducing effect of three inducers on enzyme production in several Trichoderma species. Activities of α-glucanase, β-glucanase and chitinase are determined. Furthermore, growth period optimal for enzyme production is also investigated.

Activities of three enzymes are used as indicator to select enzyme samples for further study.

The second part describes the effect of selected enzyme samples on producing Volvariella volvacea protoplast. Pleurotus sajor-caju, an edible mushroom is also used as a material to produce protoplast by enzyme digestion.

2 LITERATURE REVIEW

In the past few years, a considerable amount of work was carried out on the formation and development of spherical forms called protoplasts. The name 'protoplast' is used to describe a really naked cell, absolutely free of wall residues, and enclosed by plasma membrane only (Peberdy, 1978; Kevei, 1981). Since the first report appeared on protoplast isolation from yeasts and filamentous fungi (Peberdy, 1979a), improved methods were continuously being developed. A major line of research with fungal protoplasts has been related to their development as a system for the study of cell wall synthesis (Peberdy, 1979b). The power of wall regeneration and the reversion of protoplast to normal cell form was manifested and stimulated more studies in this field. Subsequently, protoplasts provided a useful tool in the studies on organelles (Duell *et al.*, 1964) and metabolic properties (Kuo and Lampen, 1971, Dooijewaard-Kloosterziel *et al*, 1973). However, induced fusion of the microbial protoplasts was rapidly expanding since stimulation due to the success of using protoplasts in genetics originally with higher plants. For example, the use of

microbial enzymes promoted protoplast formation from plant cells by Cocking (1960) and then let them fused (Power et al., 1970; Anné and Somer, 1976). Thus, the protoplast fusion of filamentous fungi gave an opportunity for gene transfer in both basic and applied studies (Peberdy, 1980; Ferenczy, 1981).

Enzymatic methods

Protoplasts have been released from fungi belonging to all the major taxonomic groups (Villanueva and García-Acha, 1971). The most effective method for protoplast release depends on degradation of the hyphal wall by lytic enzymes. In 1959, snail enzyme (Helix pomatia digestive juice) was the first being used (Eddy and Williamson, 1959). The snail digestive juice was shown to be effective against yeast cell walls, with the exceptions of the Rhodotorula and the Cryptococcus groups (Kevei, 1981). It was also active against several other fungi (Peberdy, 1979a), but its effectivity was more dependent upon growth conditions of the fungi. This enzyme contains a number of carbohydrases including chitinase and β-glucanase and is still used in some laboratories. Several actinomycetes such as some species of Streptomyces and Micromonspora produced enzyme complexs that broke down the cell wall of a number

of fungi (Lopez-Belmonte et al., 1966; Peberdy, 1971; Peberdy and Gibson, 1971; Peberdy, 1979a). Several Bacillus and Arthrobacter species were also studied to produce lytic activities against fungal wall (Villanueva and Gracía-Acha, 1971). Recently, many workers have found that certain species of fungi had the ability to produce enzyme complex such as in Trichoderma (Hasegawa and Nordon, 1969; Hashimoto, 1971; de Vries and Wessels, 1972; de Vries and Wessels, 1973b; Griffin et al., 1974; Peberdy and Isaac, 1976; Gong et al., 1977; Sternberg and Mandels, 1979; Sandhu and Kalra, 1982; Sternberg and Mandels, 1982).

The components of the enzyme mixtures active in breaking down fungal cell walls were known in some cases. Cellulase and laminarinase were essential for protoplast formation in Pythium (Peberdy, 1978). In the study of Schizophyllum commune by de Vries and Wessels (1973a), it was found that chitinase, α-glucanase and β-1,3-, β-1,6-glucanase were three major components of the enzyme produced by Trichoderma viride, but the first two components were essential. A wide range of enzyme combinations was found in different microorganisms, and the suitable combination for certain fungal protoplast formation was closely related to the cell

wall composition of the protoplast origin. Moreover, it was noticed that some lytic enzymes produced by microorganisms were inducible. Inducers present in growth medium was investigated in many reports (Mandels et al., 1962; Griffin et al., 1974; Price and Storck, 1975; Sandhu and Kalra, 1982). A variety of inducer has been used including homogenized fungal mycelium (Anné et al., 1974) or purified hyphal walls (de Vries and Wessels, 1972; Qiu et al., 1982). In other attempt, semi-defined medium containing polysaccharides similar to those found in fungal walls has been used. For example, chitin and laminarin were the effective inducers (Peberdy and Buckley, 1973; Laborda et al., 1974). Moreover, many workers used lytic enzyme mixed with other enzyme components to enrich the efficency (Sietsma and de Boer, 1973; Anné et al., 1974; Binding and Weber, 1974; Torres-Bauzá and Riggsby, 1980; Hashiba and Yamada, 1982). Addition of crude enzyme from Streptomyces graminofaciens and cellulase to lytic enzyme from Trichoderma harzianum increased the Penicillium chrysogenum protoplast yield (Anné et al., 1974). On the other hand, a high yield of protoplasts could be prepared from a

variety of fungi using relatively cheap commercial enzymes by Hamlyn et al. (1981).

The mycelium

Mycelial cell walls from cultures in the early and the mid-exponential phases of growth were more susceptible to lytic enzymes than walls derived from older cultures (Duell et al., 1964; Shahin, 1972; Anné et al., 1974; Deutch and Parry, 1974; Peberdy et al., 1976; Hashiba and Yamada, 1982; Santiago, 1982a). The mycelium in young stage still has not forming a complete cell wall system (Burnett, 1979) and efficency of the enzyme was closely related to the fungal wall composition, so that mycelial age was an important factor to lysis. For example, it was shown that Aspergillus nidulans hyphae was more resistant to lysis by a β-1,3-glucanase-chitinase mixture as the increase of melanin. Amount of melanin was directly correlated with the culture age (Kuo and Alexander, 1967). One of the hypothesis being advanced to account for the protection afforded by the melanin is that it may inhibit one or more of the enzymes participating in lysis of fungi (Bloomfield and Alexander, 1967). In de Vries and Wessels' study (1973b), the resistance of α-1,3-glucan to enzymatic degradation increased with culture age of Schizophyllum

commune and the yield of protoplasts was reduced. On the other hand, it was possible that the wall-bound lytic enzymes were active at exponential phase and thus enhanced the lytic effect of the enzymes (Bartnicki-Garcia and Lippman, 1972).

The facility with which young mycelia undergo lysis by lytic enzymes suggests that some changes is manifested in hyphal wall. Thus, environmental effects that also seemed causing changes in fungal cell wall were studied. For example, the nature of culture medium used was a critical factor. In 1965, Rizvi and Robertson found that disintegration of hyphal apices of a Neurospora crassa mutant, which occurs upon treatment with L-sorbose, was thought to be due to interference with the production of wall glucan. In 1968, Musikova and Fencl compared the effects of the culture of Aspergillus niger on a variety of media for protoplast release. Protoplast yields were high from mycelium growing on a glucose-salts-asparagine medium (Peberdy, 1978). Using the nitrogen source of NH_4Cl or yeast extract reduced the protoplast yeild. Some workers showed that protoplast formation from Schizosaccharomyces pombe was higher from cells grown in medium containing 2-deoxy-D-glucose (Birnboim, 1971; Foury and Goffeau, 1973).

Pretreatment of hyphal wall was also a method to enhance protoplast release. It could stimulate protoplast formation and make older mycelium as susceptible to the lytic enzymes as younger mycelium (Duell et al., 1964; Sommer and Lewis, 1971; Torres-Bauzá and Riggsby, 1980). Dooijewarrd-Kloosterziel et al. (1973) found that formation of protoplasts from Geotrichum candidum by lytic enzymes was considerably stimulated by adding thiol compounds. The effect of these compounds were related to the action of reducing S-S linkages in wall protein of the fungus. Thus, hyphal wall could be loosened at some points and this enhanced the lytic enzyme's power. Moreover, it was found that proteolytic enzyme added to the medium had the same stimulating effect on protoplast formation as had the thiol compounds. These results suggested the presence of proteinaceous material in outer layers of the hyphal wall could be removed by thiol compounds action. The ability of thiol compounds to stimulating lysis was also supported by electron microscopic study. Candida albicans cells pretreated with dithiothreitol showed only the inner electron-transparent layers and the innermost layers of the cell wall present. The outer layers were removed (Torres-Bauzá and Riggsby,

1980). For another example, pretreatment of Pythium mycelium with a detergent Triton X 100 stimulated protoplast release. It was probably because that the hyphal wall was covered with a lipid layer which could be removed by Triton X 100 (Sietsma and de Boer, 1973).

Osmotic stabilizers

In intact cells of yeast or other fungi the mechanical protection of cell wall prevents the disintegration of cells suspended in water or in very dilute medium. It is well known that if the cell wall is digested the cytoplasmic membrane disintegrates unless an osmatic stabilizer is applied. This stability can be obtained by adding to the medium a substance that does not penetrate, or penetrate very slowly, into the protoplast. The concentration of solutes used as stabilizers varies widely. This phenomenon can be correlated to some extent with differences in internal osmotic pressure of different microorganisms. A wide variety of stabilizers has been used by different workers to obtain protoplasts. It was investigated that properties of some solutes fit not only as stabilizers but also taking into consideration for other important factors, such as the stimulatory effect of the solutes on lytic enzymes and the degree of bacterial contamination

in protoplast suspension. Additionally, some relation was found between osmotic stability of protoplasts and their metabolic properties (Kuo and Lampen, 1971). Buffer containing one of the following chemicals has commonly been used. Sugars and sugar alcohols have been used successfully with yeasts and some other fungi (Bachmann and Bonner, 1959; Lopez-Belmonte et al., 1966; Somer and Lewis, 1971; Gibson and Peberdy, 1972; Hoffmann, 1981; Hashiba and Yamada, 1982). Nevertheless, inorganic salts have been proved more effective with filamentous fungi (Gascón and Villanueva, 1965; Sietsma and de Boer, 1973; Peberdy, 1979a). For example, in Sietsma and de Boer's study, several osmotic stabilizers were tested for their effect on protoplast formation of a Pythium strain. Inorganic salts ($MgSO_4$, NaCl, KCl and NH_4Cl) were very favourable when added at a suitable concentration, and organic stabilizers (sorbitol, mannitol and sucrose) strongly inhibited protoplast release. One of the inorganic salts, $MgSO_4$, can be single out for its interesting observations when used with filamentous fungi (de Vries and Wessels, 1972; Santiago, 1982a). It was observed that mycelium of Schizophyllum commune completely fragmented in the early period of lytic digestion.

Another phenomenon specific to the use of $MgSO_4$ was a process of vacuolization of the residual cytoplasm and certain protoplasts. Two classes of protoplasts were found, those with large vacuoles floated on centrifugation and those non-vacuolate and sedimented. This differential property was used as a procedure for obtaining protoplasts uncontaminated with mycelial debris.

pH value

As a suitable stabilizer system was used, pH value was also an important factor. Clearly it showed that pH value of the lytic mixture had a variable effect on protoplast yield for different species reflecting the different lytic enzyme used. For example, lytic enzyme from Trichoderma viride tested for activity against Schizophyllum commune mycelium exhibited maximum yields around pH 5.8 (de Vries and Wessels, 1972). This value was near to that used by Santiago against Volvariella volvacea (1982a). However, it varies with different enzyme systems. In Peberdy et al. (1976) study, for all the fungi examined the optimum in protoplast production was between pH 6.0 to 6.5. Clearly, there is no universal stabilizer and pH value for protoplast production and it is necessary to determine the best for each specific organism.

Enzyme concentration

The lytic activity of an enzyme preparation on protoplast release was related to concentration of the enzyme. Within the range tested, the rate of protoplast formation and the final number of protoplasts increased with increasing enzyme concentration (de Vries and Wessels, 1972; Santiago, 1982a). It was observed that higher concentration of Trichoderma lytic enzyme beyond an optimal range was unsuitable because of the lysis of most protoplasts soon after their appearance. It was probably due to high levels of proteinase in the digestive mixture (Santiago, 1982a). On the other hand, protoplast yield was found to be proportional to concentration of the mycelium used. Addition of more mycelium beyond optimum concentration reduced protoplast yield considerably (Peberdy et al., 1976; Santiago, 1982a).

Protoplasts from spores

Fluorescent nuclear staining of Volvariella volvacea protoplasts was done by Santiago (1982a) and it was shown that 42% of the early protoplasts lacked a nucleus. Total disintegration of fungal hyphal mass was closely related to this phenomenon that leading to the division of cytoplasm into discrete protoplasts. Spores of fungi would provide a most useful source of protoplast

from many filamentous fungi, since in most cases they are uninucleate structures. Reports describing the release of protoplasts from spores, however, are limited (Bachmann and Bonner, 1959; Weiss, 1965; Manocha, 1968; Villanueva and Garcia-Acha, 1971; Laborda et al., 1974, Moore and Peberdy, 1976; Bos and Slakhorst, 1981). It is probably related to the problems in obtaining protoplasts for which the spore walls are more resistant to lysis than mycelial walls in some fungi (Chu and Alexander, 1972). In general, digestion period required is longer for protoplast production from spores than that from mycelium (Laborda et al., 1974; Peberdy, 1978). In Moore and Peberdy's study (1976), using Aspergillus flavus conidia from liquid cultures, protoplast could be produced in same length of time as with mycelium. It was suggested that spore cultural conditions might affect protoplast release.

Nonenzymatic methods

Besides lytic enzyme method, the protoplast may be liberated by a procedure that has been developed for Schizosaccharomyces pombe (Peberdy, 1979a) and Histoplasma capsulatum (Berliner and Reca, 1970). High concentrations of $MgSO_4$ and small amount of 2-deoxy-D-glucose were

introduced in the culture medium. For 2-deoxy-D-glucose was a glucan synthesis inhibitor causing interference with the cell wall constitution. And, the action of $MgSO_4$ appeared to be limited to a very small highly susceptible area of the cell wall, namely, the most recently formed bud scar which might be delimited only by a membrane or an incomplete wall. However, Foury and Goffeau (1973) reported that 2-deoxy-D-glucose reduced respiratory activity of Schizosaccharomyces pombe protoplasts. Another specific method of protoplast release was demonstrated by Tyrrell and MacLeod (Peberdy, 1979a). Conidia of Entomophthora, an entomogenous fungus, was inoculated in insect tissue culture medium. The fungus conidia produced germ tubes and then released their cytoplasmic content as protoplasts. Because of their specificities for particular organisms, the above methods have not been used extensively.

Protoplast formation

The fungal hypha is generally recognized as heterogenous in organellar constitution. During protoplast formation, cytoplasm of hypha is divided into discrete protoplasts. Thus, total disintegration of a hyphal mass might be expected to have various kinds of protoplasts that are heterogeneous in their organellar

constitution and biochemical function (Peberdy, 1979a). The length of time for the first protoplast released varies with concentration of enzyme and incubation conditions. It is also depended on the combination of fungi and lytic enzyme used. In general, protoplasts appeared to be squeezed out through small pores as small round bud-like structures. These bud-like structures increased in size relatively rapidly at the expense of mycelium contents, which simultaneously decreased in size. Entire contents of a hyphal compartment were sometimes extruded as a single large protoplast. On other occasions, fungal mycelium completely fragmented and extruded intermittenly through one or several pores, giving rise to a number of protoplasts, and frequently some residual cytoplasm was left in the hypha. Less frequently, extrusion of one protoplast was followed by another of the same size connected to the first one. On the other hand, it was found that in treatment with very active enzyme solution masses of protoplasts were left closely adpressed to each other (Peberdy, 1971). In suitable condition, the hyphae were extensively digested and showed big pores or holes all along cell wall after long incubation. Observation of protoplast from Fusarium culmorum by

Lopez-Belmonte *et al.* (1966) showed that some cytoplasmic contents passed through septal pore of the hypha. It was found some thin thread connecting the contents of two neighbouring cells. The thread was sometimes included small spherical structure of cytoplasm. Nevertheless, protoplasts released from conidia only originated through small pores that developed in spore walls. As a rule, the entire contents of one conidium were extruded through one pore on side wall to form one free protoplast (Manocha, 1968). It was found that physical forces might increase the rate of protoplast release. In the study of *Fusarium culmorum* (Lopez-Belmonte *et al.*, 1966), when incubation mixture was diluted with water just at the moment when protoplasts started to extrude, liberation of protoplasts took place much more quickly.

Protoplasts were sensitive to osmotic shock. When placed in distill water they swelled slightly and burst suddenly. Vacuoles presented in protoplasts were remained in sight for some time. When placed in hypertonic solutions they shrank quickly. When shrunken protoplasts were subsequently placed in distill water, they swelled and burst. In the presence of osmotically stabilized solution, protoplast could be stable for a period of time. However, at a very high concentration

of stabilizer mycelium may become completely plasmolysed and cytoplasm was divided into small pieces which were not released from the mycelium.

Protoplasts were not uniform. They were of different sizes. Contents of protoplasts were also not uniform. In appearence, some protoplasts were refractile and others were quite dark. The different observations related to the presence or absence of vacuoles, ribosomal density and numbers of organelles in protoplasts. Number of vacuoles per protoplast varied. Some had a very large vacuole or several vacuoles, whereas others had none or they were very small. When $MgSO_4$ was used as an osmotic stabilizer, large vacuoles which accounted for the greater part of protoplast volume could be seen (de Vries and Wessels, 1972). For example, there were some detail studies on Aspergillus nidulans protoplasts (Peberdy and Gibson, 1971). Small and non-vacuolate protoplasts were released first during incubation of mycelium in a lytic enzyme and larger vacuolate ones were produced after 3 h. Electron-microscopic study showed the original observations at an ultrastructural level (Gibson and Peberdy, 1972). The early released protoplasts were small and dense ribosome-rich cytoplasm and small vesicles were present. The second type of

protoplasts released later was larger, vacuolate and had diffusely granular cytoplasm with fewer ribosomes. The difference in the time of release of protoplasts might be caused by differences in thickness of hyphal walls, which increases with distance from hyphal tip. Thus, some protoplasts were released from young hyphal tip early and others, released later, originated from regions progressively further from hyphal tip. Based on preceding information and literature cited, a model for the formation of new wall at hyphal tip can be outlined. An early step is the incorporation of wall subunits, lytic enzymes, and synthetic enzymes into vesicles. The vesicles are then attaining in contact with the plasma membrane at the apex of mycelium. The two membranes fuse, thus depositing the vesicle contents into the wall region at the apex (Grove, 1978). The occurence of small vesicles in protoplasts released early in incubation might therefore suggest that those protoplasts originated from apex of a hypha. Protoplasts released later, probably from older regions of the hyphal filament, showed the absence of small vesicles and the presence of vacuoles. It is generally considered that cytoplasmic streaming is caused by vacuolation of distal hyphal regions forcing cytoplasm towards the apex, thus

the tips remain in a constantly turgid state without vacuoles. These considerations would explain the differences in ribosome density and cytoplasmic vacuolation in protoplasts released early and later during incubation.

Properties of Protoplasts

The metabolic properties of fungal protoplasts have not been extensively studied. In the study of Fawcett et al. (1973), protoplasts of Penicillium chrysogenum and Cephalosporium acremomium showed the same metabolic activities as intact mycelium. Study of Aspergillus nidulans had another finding. Protoplasts produced with KCl showed an increase in respiratory activity over the mycelium for 3 h incubation, whereas those in $MgSO_4$ had an activity that was much higher at the first hour of lytic digestion (Peberdy, 1979a). Scarborough and Schulte (1974) compared the transport systems of Neurospora crassa protoplasts and showed that they were unaffected after removal of the hyphal wall. The secretion of macromolecules, including enzymes, is an important aspect of the biology of fungi. Kuo and Lampen (1971) carried out a detailed analysis of invertase synthesis and secretion by protoplasts from Saccharomyces. Osmotic pressure had a marked influence on the metabolic

functions of protoplasts. Invertase formation was inhibited at high osmolarity. However, the reduction was eliminated when protoplasts were transferred into a medium of lower osmolarity. The rate of fructose uptake was decreased by high osmolarity, also reduction of invertase formation could be partially reversed by increasing the level of sugar supplied as energy source.

3 ENZYME PRODUCTION

3.1 Introduction

The imperfect fungus, Trichoderma belongs to Moniliaceae of Moniliales. This genus was first introduced by Persoon in 1794 (Rifai, 1969). In 1902, the first report on the occurrence of Trichoderma species in soil was published by Oudemans and Koning (Rifai, 1969). They obtained an isolate named Trichoderma koningii Oudemans. The genus was first treated monographically by Rifai (1969) in which emphasis was laid on the microscopic characters. It was based on the types of branching system of conidiophores, the manner of phialide disposition and the characters of the phialospores. Macroscopic appearance of colony, e.g. growth rate of colonies, color of colonies and pigment secreted into the medium, were also used in classifying these fungi. Nine species were recognized by Rifai, which included four classical ones (Trichoderma hamatum (Bon.) Bain., T. koningii Oud., T. viride Pers. ex S. F. Gray and T. polysporum (Link ex Pers.) Rifai) and five new species (T. piluliferum Webster & Rifai, T. aureoviride Rifai, T. harzianum Rifai, T. longibrachiatum

Rifai and T. pseudokoningii Rifai).

With increasing interest in fungi living in soil, extensive literatures that dealt with the distribution of soil fungi were reported and it showed substantially that species of Trichoderma were numerous and widely distributed. For example, in Widden and Parkinson's studies (1973), pine forest soils in Canada tended to have Trichoderma species as the dominant fungi. In Sweden, Söderström (1975) indicated that Trichoderma polysporum and T. viride were very abundent in a number of spruce forests. During a study on ecology of forest-soil fungi in Southern Quebec (Widden, 1979), species of Trichoderma were found at all areas investigated. The results, however, showed that species of these fungi had distinct patterns of distribution with respect to the type of tree established. Moreover, Widden and Abitbol investigated the effects of seasonal changes on Trichoderma population (1980). It was showed that Trichoderma viride and T. polysporum were generally found at cooler periods of the year and T. koningii occured during the warmer period.

Many ecological studies revealed that Trichoderma played a very important role in soil ecosystem. On the other hand, as shown by Komatsu's study, the most important

edible mushroom in Japan was attacked by Trichoderma species during its cultivation on the bed-logs (1976). Thus, researches were undertaken to know more about the physiological behavior of this fungus. In Komatsu's study, mycelial development and conidia germination had some relationships to temperature and humidity (1969). Sporulation in Trichoderma viride was inducible with illumination. When culture was reset in the dark, sporulation was suppressed without greatly affecting its growth (Galun and Gressel, 1966). The detectable quantum efficiency was between 350 nm and 550 nm, and no spore formation occured at 254 nm or from 525 to 1100 nm (Gressel and Hartmann, 1968). Moreover, both carbon and nitrogen sources were found to be necessary for the germination of Trichoderma conidia (Aube and Gagnon, 1969; Martin and Nicolas, 1970; Mitchell and Dix, 1975).

In 1932, Weindling reported the parasitic action of Trichoderma species on Rhizoctonia solani and other soil fungi (Weindling and Emerson, 1936). Coiling by hyphae of this fungus around hyphae of the host was described. Vacuolation, coagulation of cytoplasm and sometimes bursting of the host hyphae were observed. Dennis and Webster mentioned that cellulolytic activity of the fungus might enable its hyphae to penetrate host hyphae

and caused the above result (1971). Since antagonistic activity of Trichoderma stimulated attempts to obtain biocontrol system, some workers attempted to study the mechanism of interaction between Trichoderma and its hosts (Rodriguez-Kabana and Curl, 1968; Rodriguez-Kabana, 1969). However, it was found that Trichoderma species appeared to posses necesary enzymes to attack fungal walls composed of chitin, glucan and xylan polymers, as well as cellulose (Dennis and Webster, 1971). Simple, efficient and economical processes for conversion of cellulose to glucose by enzymatic hydrolysis would aid in developing new food sources (Mandels et al., 1971). In addition, the fungus was particularly suitable for controlled fermentation of animal wastes to decrease pollution level by concentrating waste and increase crude protein content of residue (Griffin et al., 1974). It was well established that the cellulolytic system of Trichoderma was the most suitable for saccharification of all kinds of cellulosic materials. On the other hand, Trichoderma species were known to excrete β-glucosidase, xylanase, pectinase, cellobiase, etc. (Hashimoto, et al., 1971; Gong et al., 1977; Rodriguez-Kabana et al., 1978; Herr, 1979; Sandhu and Kalra, 1982; Sternberg and Mandels, 1982). Recent studies on the protoplast formation

stimulated the use of lytic enzymes preparation obtained from microbial origins. Thus, Trichoderma species were much more effective in producing protoplasts (de Vries and Wessels, 1972; Peberdy and Isaac, 1976; Santiago, 1982b). In 1972, de Vries and Wessels reported an effective enzyme preparated from Trichoderma viride grown on hyphal walls of Schizophyllum commune. They found the lytic enzyme had the ability to attack S-glucan (α-1,3-linked glucan) in cell wall of S. commune hence to release protoplasts. In the next year, the same research group examined other two wall polymers, R-glucan (β-glucan) and chitin of Schizophyllum commune. S-glucanase, R-glucanase and chitinase were purified from the Trichoderma enzyme mixture (de Vries and Wessels, 1973a). Another species of Trichoderma, T. harzianum, was also useful in producing lytic enzyme mixture (Peberdy and Isaac, 1976; Isaac et al., 1978; Santiago, 1982b). In Qiu Jingyun et al. studies, Trichoderma longibrachiatum was used (1982).

It was considered that some components of lytic enzyme mixture from Trichoderma were formed in the presence of inducers. As research workers wish to obtain protoplasts from certain organisms, information of main components of their cell walls are important. Hyphal walls of

filamentous fungi generally contain chitin and glucan units (Burnett, 1979; Peberdy, 1981). Mycelia or hyphal walls of fungi were used as inducers, chitin was also employed. Improved efficiency for the lytic enzyme from Trichoderma harzianum was reported, probably due to the addition of some inducers(Peberdy and Isaac, 1976; Santiago, 1982a). β-glucans occurred in micro-organisms and higher plants as structural constituents of cell walls, as reserve materials and as extracellular substances of uncertain significance (Chesters and Bull, 1963a). Laminarin, a glucose polymer composed entirely of β-D-glucopyranose with 1,3-linkages, was first described by Schmiedeberg(1885), who isolated it from the Laminariaceae. It was also an useful inducer. Laminarin occurred only in frond of the Laminariaceae and to a lesser extent in the Fucaceae, and was subject to marked seasonal variations (Black et al., 1951; Peberdy and Isaac, 1976). Because pure laminarin was very expensive, it was replaced by Laminaria meal (Peberdy and Isaac, 1976). Fronds of Laminaria hyperborea and L. digitata were collected in autumn. After washing and drying, they were finely ground prior to use.

Experiments were carried out to examine the inducing effect of some substances on enzyme production in

Trichoderma. The inducers included *Volvariella volvacea* hyphal wall, *Laminaria* meal and chitin.

3.2 Materials and Methods

3.2.1 Fungal materials

Five stock cultures of *Trichoderma* species in complete medium were obtained from Prof. S. T. Chang, Department of Biology, The Chinese University of Hong Kong. It included two strains of *Trichoderma harzianum* (T1 and T9), a strain of *T. longibrachiatum* (T6) and a strain of *T. koningii* (T2). TX was a *Trichoderma* strain isolated from cotton waste compost for cultivation of straw mushroom, *Volvariella volvacea*. Compost sample was collected before harvesting. Samples were collected and suspended in sterile water in a 100 ml conical flask. This suspension was then shakened and pipetted into plates. The medium used was complete medium. The plates were incubated at room temperature ($\pm$ 25°C) under constant light illumination. After 2 days, isolated colonies were obtained. All the *Trichoderma* species used were grown in basic TLE agar medium plus 0.5% (W/V) chitin and 1% (W/V) *Laminaria* meal at room temperature ($\pm$ 25°C) under illumination.

Volvariella volvacea V5 was obtained from Prof. S. T. Chang. The organism was maintained in complete

medium agar plate at 28°C.

3.2.2 Culture medium

Trichoderma species was cultured in basic TLE medium containing (g/L): Bactopeptone (BDH), 1 g; urea, 0.3 g; KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 1.4 g; $MgSO_4 \cdot 7H_2O$, 0.3g; $CaCl_2 \cdot 2H_2O$, 0.3g; trace elements solution, 1 ml (Vogel, 1964); glucose, 3 g (autoclaved separately and added to the medium when cool); and agar, 15 g. (Add distill water to make a liter)

Volvariella volvacea V5 was maintained in complete medium containing (g/L): $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.46 g; K_2HPO_4, 1 g; peptone (BDH), 2 g; glucose, 20 g; yeast extract (Difco), 2 g; thiamine HCl, 0.5 mg; and agar, 20 g. (Add distill water to make a liter)

3.2.3 Inducer preparation

For induction studies, Volvariella volvacea V5 hyphal wall, Laminaria meal and chitin were used.

The hyphal wall of V5 was prepared from cultures grown in liquid complete medium at 28°C for 7 days. Cultures were incubated in a rotary shaker (150 rotation per min). The mycelium was harvested by vacuum filtration,

washed three times with distill water and then autoclaved for 15 min. After further washing the material was freeze-dried and finally ground to fine powder with a grinder (Fritsch-pulverisette) (Santiago, 1982a). Five grams of this material were added to one liter of basic TLE medium.

The Laminaria meal was prepared by dried Laminaria fronds bought at local market. After washing, the fronds were dried at 80°C and then finely ground prior to use. Ten grams of the meal were added to one liter of TLE medium.

Chitin (Poly-N-acetylglucosamine) from crab shells manufactured by Sigma Co. was used as inducer (5 g/L).

3.2.4 Methods

3.2.4.1 Production of lytic enzyme

Medium used for the study was basic TLE liquid medium. In order to determine which inducers are needed for optimal lytic enzyme production, basic TLE plus various components were prepared as shown in Table 3.1.

Mycelium culture of Trichoderma was grown in basic TLE agar medium plus 1% Laminaria meal and 0.5% chitin

in 9 cm petri dishes at room temperature (± 25°C) under constant illumination for 7 days. The conidia were suspended in autoclaved distill water and proper dilution was made for a final concentration of approximately 1 X 10^6 conidia per ml. Each 500 ml of medium was placed in a 2000 ml Erlenmeyer flask. After inoculated with conidia suspension of described concentration, the flask was incubated at 28°C in a rotary shaker at 150 rpm (rotation per min) under constant illumination.
The day started to incubate was day 0. The entire contents of the flask was harvested by centrifugation (10,000 g for 30 min at 6°C). The supernatant was added with $(NH_4)_2SO_4$ to 75% saturation at 4°C. After centrifugation, the precipitate was taken up in water and, after dialysis for 24 h against several changes of distill water, insoluble material was removed by centrifugation. The enzyme solution was then lyophilized and stored.

3.2.4.2 Protein test

Protein was determined with a modified method by Lowry et al. (1951). The reagents were prepared freshly by the addition of 1% $CuSO_4 \cdot 5H_2O$ mixed with 2% sodium

potassium tartrate, then added 2% Na_2CO_3 in 0.1 M NaOH solution at a proportion of 1:1:100. One ml of sample was mixed with 5 ml of reagent and allowed to stand for 10 min at room temperature (± 25°C). Half ml of Folin-Ciocalteau phenol reagent (Sigma, freshly diluted with distill water to 1 N) was added very rapidly and mixed instantly. After 30 min, the sample was read in a spectrophotometer at 750 nm. Bovine serum albumen (Sigma) was used as a standard.

3.2.4.3 Enzyme assay methods

3.2.4.3.1 α-glucanase assay

Substrate: 0.1% nigeran (Koch-light) (W/V) in 0.2 M phosphate buffer, pH 5.8.

Enzyme assay:

Activity was determined by measuring the release of glucose in a reaction mixture using a modified method of Huotari *et al.* (1968). Assay mixture comprised 0.1 ml of enzyme solution at a suitable concentration in 0.01 M phosphate buffer (pH 5.8) and 0.9 ml of substrate. It was incubated in a water bath at 37°C. After 1 h, insoluble nigeran was removed by centrifugation to prevent

turbidimetric interference with colorimeter readings (Hasegawa and Nordin, 1969). Ten g of DNS (3,5-dinitrosalicylic acid) were dissolved in about 500 ml distill water. Ten g of NaOH and 200 g of Rochelle salt (sodium potassium tartrate) were added and warmed to dissolve. Finally, 2 g of phenol and 0.5 g of Na_2SO_3 were added. The solution was heated and stirred to dissolve the chemicals, and then added distill water to make 1 liter. The solution was storaged in color bottle at $4^{o}C$. Three ml of the prepared DNS were added to the enzyme-substrate mixture and heated in vigorously boiling water for 15 mins. It was cooled in tap water immediately and read in a spectrophotometer at 550 nm. A standard curve, linear in the range of 0-2 μ moles$\cdot ml^{-1}$ glucose was used.

3.2.4.3.2 β-glucanase assay

Substrate: 0.05% laminarin (Sigma) (W/V) in 0.2 M phosphate buffer, pH 5.8.

Enzyme assay:

The mixture comprised 0.1 ml enzyme solution at a suitable concentration in 0.01 M phosphate buffer (pH 5.8) and 0.9 ml substrate. It was incubated in a water bath

at 37°C for 30 min. The assay procedure was the same as in α-glucanase assay using DNS as reagent.

3.2.4.3.3 Chitinase assay

Substrate: Commerical chitin was converted to the acid swollen form before use. Twenty g of chitin were added to 500 ml concentrated HCl at 4°C with stirring. After filtration through glasswool, the solution was poured slowly into 50% aqueous ethanol solution with constant stirring. The precipitate was washed thoroughly with distill water until neutralized, freeze dried and then ground to fine powders (Skujins, Potgieter and Alexander, 1965; Peberdy, 1981).

Enzyme assay:

The assay for N-acetylglucosamine in the supernatant after enzyme reaction was determined by a modified method of Reissig, et al. (1955). Assay mixture comprised 0.5 ml enzyme solution at a suitable concentration in 0.01 M phosphate buffer (pH 5.8) and 0.5 ml suspension of 25 mg chitin in 0.02 M phosphate buffer (pH 5.8). The reaction mixture was incubated in a water bath at 30°C for 1 h,

and then added 0.2 ml of 0.8 M potassium tetraborate adjusted to pH 9.1 with boric acid. It was heated in vigorously boiling water bath for 3 mins. After cooling down quickly, the reaction mixture was centrifuged. Ten g of DMAB (p-dimethylaminobenzaldehyde)**were** dissolved in 100 ml AR glacial acetic acid (12.5% (V/V) 10 N HCl), storaged at 2°C, shortly before use it was diluted with 9 volumes of acetic acid. The centrifuged supernatant of 0.6 ml was mixed with 3 ml of the prepared DMAB immediately and incubated in a water bath at 36-38°C. After 20 mins the mixture was cooled in tap water and read in a spectrophotometer at 585 nm. A standard curve, linear in the range 0-0.25 μ moles·ml^{-1} N-acetylglucosamine, was used.

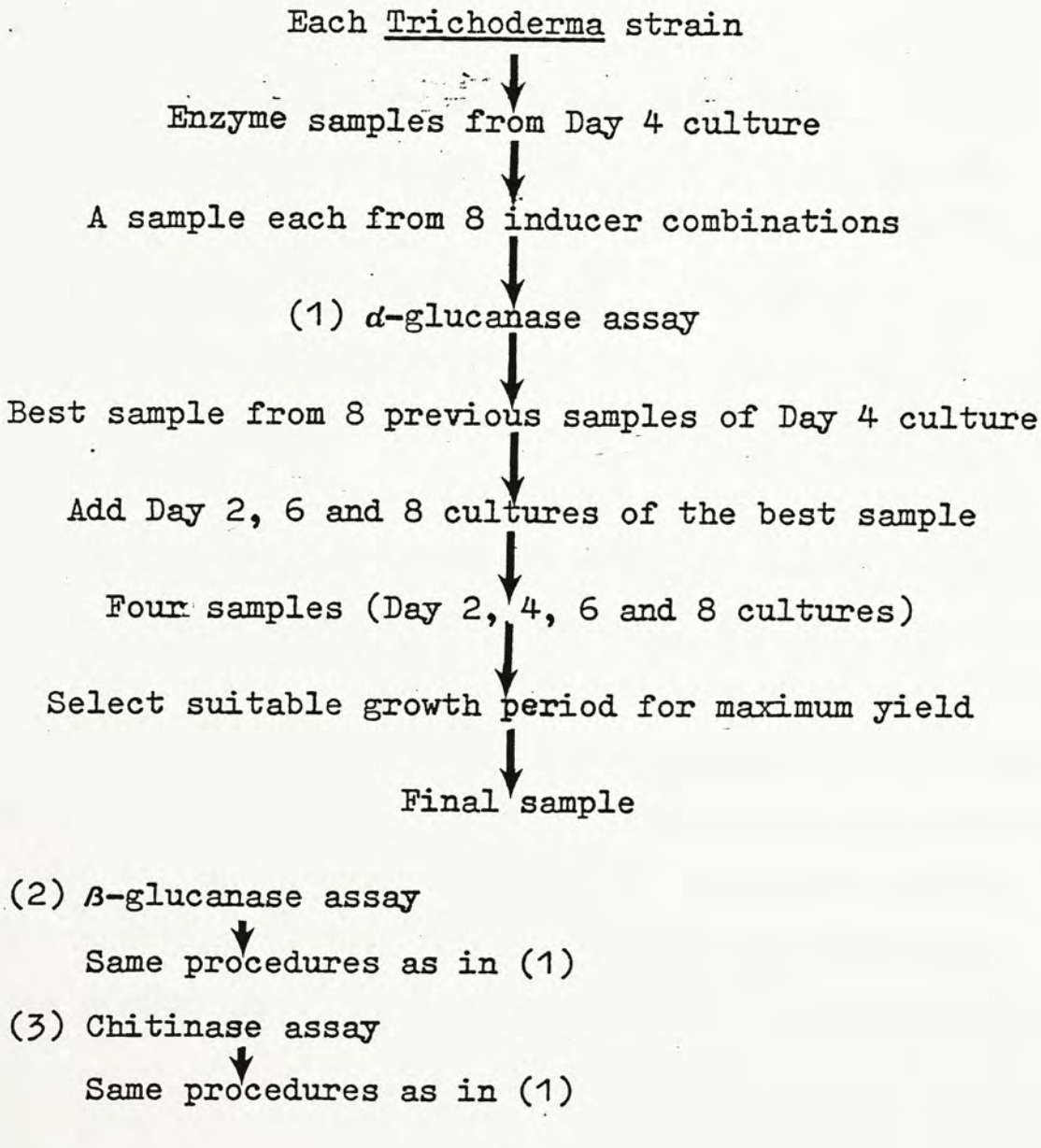
Table 3.1 Various components of inducers added

A.	TLE medium plus single inducer
	0.5% V5 hyphal wall
	1% *Laminaria* meal
	0.5% chitin
B.	TLE medium plus double inducers
	0.5% V5 hyphal wall and 0.5% chitin
	0.5% V5 hyphal wall and 1% *Laminaria* meal
	0.5% chitin and 1% *Laminaria* meal
C.	TLE medium plus triple inducers
	0.5% V5 hyphal wall, 0.5% chitin and 1% *Laminaria* meal
D.	No inducer (basic TLE medium only)

3.3 Scheme

Enzyme activity was used as a tool to screen the samples from various combination of fungal species, inducers used and growth periods. The scheme was shown in Fig. 3.1.

Fig. 3.1. Selection of enzyme samples



3.4 Results

3.4.1 Variation in α-glucanase production with different inducer combinations

Basic TLE medium plus various inducer components were prepared as in Table 3.1. Results of five *Trichoderma* strains were shown in Tables 3.2 to 3.6. The samples were collected from Day 4 culture.

It had no correlation in the enzyme formation between T1 and T9 although they are the same species (*Trichoderma harzianum*) (Tables 3.2 and 3.3). V5 hyphal wall plus *Laminaria* meal was the best combination to induce α-glucanase in T1 (Table 3.2). The activity was about seven times greater than that of the control with only basic TLE medium. It was found that in the presence of *Laminaria* meal, α-glucanase activity was higher than others. The enzyme activity was very low when there was no inducer added. Effect of growth period on the enzyme production with V5 hyphal wall and *Laminaria* meal added was shown (Fig. 3.2). Longer incubation period generally allowed for maximum accumulation of this enzyme in the culture filtrate. However, β-glucanase and chitinase had a maximum yield on Day 6 and Day 4 respectively in the

same medium condition (Figs. 3.3 and 3.4). T9 (Trichoderma harzianum) varied in α-glucanase production with different inducer combinations (Table 3.3). Unlike T1, addition of all three inducers (V5 hyphal wall, Laminaria meal and chitin) to the culture medium enhanced enzyme production substantially. Other combinations of inducers showed no significant difference to the control. It was found that α-glucanase reached its maximum yield on Day 6. It was longer than that of both chitinase and β-glucanase which reached their maximum yields on Day 4 with all three inducers (Figs. 3.5 to 3.7).

Result of T2 (Trichoderma koningii) in producing α-glucanase at different medium conditions was listed in Table 3.4. Laminaria meal plus chitin was the best supplement to induce enzyme formations. It was found with rather similar reactions in T2 and T1. α-glucanase activity was high as Laminaria meal was supplemented as one of the components or even alone. As Laminaria meal plus chitin were added to the medium, the enzyme activity of T2 reached its maximum level in Day 6 culture, and dropped down since then (Fig. 3.8). However, activities of β-glucanase and chitinase in Day 6 culture were 80% and 84% respectively of that in Day 4 culture, which only retained 73% and 42% respectively of the maximum activity

in Day 8 culture (Figs. 3.9 and 3.10).

Activity of α-glucanase from T6 (Trichoderma longibrachiatum) in medium without Laminaria meal did not show great difference to the control, but that from the medium supplemented with Laminaria meal showed significant high in activity. Basic TLE medium with all the three inducers supplemented had a maximum yield (Table 3.5). The activity of α-glucanase present in the above medium certainly increased and then decreased in Day 6 culture (Fig. 3.11). Effects of growth period on β-glucanase and chitinase production from T6 in the presence of all three inducers were shown in Figs. 3.12 and 3.13. From these graphs it was observed that during the first 4 days, β-glucanase activity was directly proportional to the incubation time. For an incubation longer than 4 days, the enzyme lost part of its activity. Chitinase also had similar result.

TX, a Trichoderma strain isolated from cotton waste compost for cultivation of straw mushroom, significantly increased in producing α-glucanase as Laminaria meal was alone or one of the substances added to basic TLE medium (Table 3.6). It clearly showed that the activity of α-glucanase increased for longer growth period in the experiment (Fig. 3.14). The activities of β-glucanase and

chitinase increased considerably with prolonging growth period up to 4 days. Older culture beyond this period lost part of their enzyme activities(Figs. 3.15 to 3.16).

Responses of Trichoderma strains in producing α-glucanase were analyzed in Table 3.7. It revealed that high levels of α-glucanase were produced by the fungus when Laminaria meal was one of the substances added. When the fungus was grown in basic TLE medium, only small amount of α-glucanase could be detected in Day 4 culture. V5 hyphal wall had no direct effect on α-glucanase formation. From the five Trichoderma strains investigated, TX culture in medium supplemented with Laminaria meal and chitin had a higher enzyme activity than others.

Maximum formation of β-glucanase and chitinase was generally observed in Day 4 culture (Figs. 3.9 to 3.16). After then, both enzymes showed a rapid drop in activity. However, α-glucanase reached its high level in still later stage.

3.4.2 Variation in β-glucanase production with different inducer combinations

For comparison of each inducer combination, data

was taken from Day 4 culture. Results were shown in Tables 3.8 to 3.12.

It was found that small amount of β-glucanase was produced by T1 (Trichoderma harzianum) in the presence of Laminaria meal (Table 3.8). The fungus produced up to about double that of the control which grown on V5 hyphal wall only. The inducing effect of V5 hyphal wall was nullified when chitin was added to it. When the carbon source for growth was glucose only, the fungus still produced β-glucanase. It was showed that maximum yield of the enzyme was harvested in Day 4 culture (Fig. 3.17). Chitinase formation had a similar result in the presence of V5 hyphal wall (Fig. 3.19). α-glucanase production from T1 in the presence of V5 hyphal wall reached its maximum yield in Day 6 culture (Fig. 3.18).

In the absence of V5 hyphal wall, activity of the enzyme from T9 was low (Table 3.9). It seemed that Laminaria meal and chitin had no direct effect on inducing β-glucanase when they were added singly or not. When Laminaria meal or chitin combined with V5 hyphal wall, they acted in part to counteract some inducing effect of V5 hyphal wall. When they were added together, the interference was reduced. β-glucanase activity was high when V5 hyphal wall was added to the medium. The enzyme

activity in different growth periods with V5 hyphal wall as an inducer was shown in Fig. 3.20. The enzyme activity was high in Day 2 culture, and reached a maximum yield on Day 4. After that growth period, β-glucanase activity declined slowly. The highest α-glucanase activity in the experiment was recorded in Day 8 culture (Fig. 3.21). The highest activities of chitinase and β-glucanase appeared similar during first 4 days incubation, after which the activity of chitinase rapidly declined in the case with V5 hyphal wall (Fig. 3.22).

Different effects of inducers on producing β-glucanase by T2 (Trichoderma koningii) were listed in Table 3.10. It was found that the enzyme activity was high when glucose was the only carbon source in medium. Laminaria meal partially inhabited the enzyme production. As comparing with α-glucanase production by the same fungus, it was showed that the conditions favored to α-glucanase formation were not suitable for inducing β-glucanase. There was a sharp increase in β-glucanase activity during the first 4 days of incubation when V5 hyphal wall and chitin were supplemented (Fig. 3.23). However, the maximum α-glucanase activity achieved on that medium appeared on Day 6 (Fig. 3.24). The maximum amount of chitinase was produced on Day 4 which was closely followed by the

α-glucanase activity (Fig. 3.25).

It was found that small amount of β-glucanase was produced by T6 (Trichoderma longibrachiatum) in the absence of any inducing substance used in the experiment (Table 3.11). As the three inducers supplemented singly to basic TLE medium, no significant effect was found. Adding all the three inducers to the medium increased the enzyme activity three times than that of the control. When using only V5 hyphal wall and chitin, the activity increased nearly 6 times than that of the control. Day 4 culture supplemented with V5 hyphal wall and chitin yielded maximum harvest of β-glucanase and then declining (Fig. 3.26). α-glucanase activity in same medium condition was quite low until Day 4 but in Day 6 culture it showed a high level of the activity (Fig. 3.27). In the same medium, chitinase activity was recorded (Fig. 3.28). The growth period in which the enzyme exhibiting very high activity was rather short. It varied from Day 2 to Day 6. The highest activity was attained in Day 4 culture.

It was shown that basic TLE medium supplementing with only Laminaria meal or other inducers had a positive effect on inducing β-glucanase production by TX (Table 3.12). The fungus secrected a small amount of the enzyme

when glucose was used as the carbon source. All the three inducers added to the medium had a significant effect on the enzyme production. The variation in β-glucanase formation with different inducer combinations by this fungus was similar to the variation in α-glucanase production. It was found that β-glucanase, α-glucanase and chitinase activities reached their maximum yields in Day 4 culture (Figs. 3.29 to 3.31). The activities of β-glucanase and chitinase rapidly declined after 4 days incubation, where the reducing rate of α-glucanase was slightly delayed until Day 6 and then dropped down quickly.

The data in Tables 3.8 to 3.12 were further analyzed (Table 3.13). Unlike α-glucanase, different Trichoderma strains had various responses in producing β-glucanase under different medium conditions. Except T6, other Trichoderma strains had the ability to produc β-glucanase when glucose was the only carbon source.

3.4.3 Variation in chitinase production with different inducer combinations

Results of the five Trichoderma strains were shown in Tables 3.14 to 3.18. The data was taken from Day 4

culture.

Different effects of inducers in producing chitinase by T1 (Trichoderma harzianum) were listed in Table 3.14. Either V5 hyphal wall or chitin added to the medium had a slight effect on inducing the enzyme. When the two inducers supplemented at the same time, some interference might be imposed. The inducing effect of the two inducers became weak when Laminaria meal was added. The effects of growth period on enzymes production in the presence of V5 hyphal wall, which produced the highest yield of chitinase, were shown in Figs. 3.17 to 3.19.

The inducer effect of forming chitinase by T9 (Trichoderma harzianum) was listed in Table 3.15. It was found with some similarities to T1. V5 hyphal wall had a positive effect on inducing chitinase. It was ten times greater in enzyme activity than that of the control. In the presence of Laminaria meal, the efficiency of V5 hyphal wall was retarted. However, chitin stimulated only slightly the fungus to produce chitinase. T9 had a low level of chitinase production in basic TLE medium when it was compared with T1. In the presence of V5 hyphal wall, α-glucanase, β-glucanase and chitinase activities in different growth periods by T9 were shown in Figs. 3.20 to 3.22.

T2 (Trichoderma koningii) varied in chitinase production with different inducers (Table 3.16). With the inducers supplemented separately to the medium there was no significant effect. The inducing effect increased when Laminaria meal and V5 hyphal wall were added separately to chitin. However, the fungus could produce chitinase when there was no inducer added. Chitin plus Laminaria meal could stimulate a higher level of enzyme production. Samples in the medium condition from different growth periods were tested. Activities of the three enzymes were shown in Figs. 3.8 to 3.10.

When basic TLE medium supplemented with only V5 hyphal wall or chitin, there was no inducing effect on chitinase production by T6 (Trichoderma longibrachiatum) (Table 3.17). But addition of both inducers was the best combination to induce chitinase. It was also the best condition for T6 to produce β-glucanase. The effects of growth period on formation of the three enzymes in described medium were listed in Figs. 3.26 to 3.28.

Chitin had a slight effect to TX on inducing Chitinase (Table 3.18). When V5 hyphal wall was added singly or combined with other inducers, the enzyme activities were retarded slightly. Similar to T2,

TX could produce chitinase even when no inducer was introduced. As chitin plus Laminaria meal was one of the conditions which attained higher chitinase yield, effect of growth period was tested (Figs. 3.14 to 3.16).

Chitinase activity of different Trichoderma strains with different inducer combinations was shown in Table 3.19. It was found that there was no significant effect when chitin was the inducer added. Laminaria meal had similar result. When both of them were supplemented, chitinase was induced in T2 and TX. The highest chitinase activity was recorded in the presence of V5 hyphal wall with two strains of Trichoderma harzianum, T1 and T9, but no stimulating effect on T6. In basic TLE medium culture on Day 4, the enzymes from T9 and T6 were quite low as compared with T1, T2 and TX.

3.4.4 Variation of mycelium dry weight and pH value in basic TLE medium

It was found that maximum dry weight of Trichoderma mycelia were generally reached in Day 2 culture on basic TLE medium (Fig. 3.32). It was also shown that pH value of the medium increased during the culture period (Fig. 3.33). The pH value in Day 8 culture was around 7.

Table 3.2: *d*-glucanase activity of T 1 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0191 ± 0.0011 bcd*
V 5 hyphal wall and *Laminaria*	0.0426 ± 0.0024 a
Laminaria and chitin	0.0213 ± 0.0033 bc
Laminaria	0.0262 ± 0.0122 b
V 5 hyphal wall and chitin	0.0066 ± 0.0006 cd
V 5 hyphal wall	0.0081 ± 0.0002 cd
Chitin	0.0257 ± 0.0003 b
No inducer	0.0059 ± 0.0003 d

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.3: α-glucanase activity of T 9 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0344 ± 0.0051 a*
V 5 hyphal wall and *Laminaria*	0.0191 ± 0.0015 b
Laminaria and chitin	0.0219 ± 0.0044 b
Laminaria	0.0161 ± 0.0001 b
V 5 hyphal wall and chitin	0.0151 ± 0.0009 b
V 5 hyphal wall	0.0215 ± 0.0034 b
Chitin	0.0208 ± 0.0066 b
No inducer	0.0141 ± 0.0003 b

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.4: α-glucanase activity of T 2 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0249 ± 0.0001 abc*
V 5 hyphal wall and *Laminaria*	0.0262 ± 0.0058 abc
Laminaria and chitin	0.0511 ± 0.0029 a
Laminaria	0.0435 ± 0.0065 ab
V 5 hyphal wall and chitin	0.0151 ± 0.0005 bc
V 5 hyphal wall	0.0085 ± 0.0011 c
Chitin	0.0065 ± 0.0005 c
No inducer	0.0071 ± 0.0001 c

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.5: *d*-glucanase activity of T 6 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0506 ± 0.0074 a*
V 5 hyphal wall and *Laminaria*	0.0307 ± 0.0003 bc
Laminaria and chitin	0.0277 ± 0.0002 c
Laminaria	0.0375 ± 0.0005 b
V 5 hyphal wall and chitin	0.0151 ± 0.0004 d
V 5 hyphal wall	0.0062 ± 0.0011 de
Chitin	0.0028 ± 0.0021 e
No inducer	0.0086 ± 0.0001 de

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.6: α-glucanase activity of T x (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, Laminaria and chitin	0.0699 ± 0.0001 b*
V 5 hyphal wall and Laminaria	0.0928 ± 0.0229 ab
Laminaria and chitin	0.1202 ± 0.0048 a
Laminaria	0.0845 ± 0.0008 b
V 5 hyphal wall and chitin	0.0061 ± 0.0001 c
V 5 hyphal wall	0.0055 ± 0.0003 c
Chitin	0.0075 ± 0.0001 c
No inducer	0.0123 ± 0.0008 c

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.7: Responses of five Trichoderma strains to different inducer combinations (V:V 5 hyphal wall; C:Chitin; L:Laminaria meal) in producing α-glucanase (μ moles glucose/min/mg protein)

T 1	(V+L)	(L)	(C)	(L+C)	(L+V+C)	(V)	(V+C)	None
	0.042	0.026	0.025	0.021	0.019	0.008	0.006	0.005
T 9	(L+V+C)	(L+C)	(V)	(C)	(V+L)	(L)	(V+C)	None
	0.034	0.021	0.021	0.020	0.019	0.016	0.015	0.014
T 2	(L+C)	(L)	(V+L)	(L+V+C)	(V+C)	(V)	None	(C)
	0.051	0.043	0.026	0.024	0.015	0.008	0.007	0.006
T 6	(L+V+C)	(L)	(V+L)	(L+C)	(V+C)	None	(V)	(C)
	0.050	0.037	0.030	0.027	0.015	0.008	0.006	0.002
T X	(L+C)	(V+L)	(L)	(L+V+C)	None	(C)	(V+C)	(V)
	0.120	0.092	0.084	0.069	0.012	0.007	0.006	0.005

* Means within underlined were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.8: β-glucanase activity of T 1 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.1624 ± 0.0058 e*
V 5 hyphal wall and *Laminaria*	0.3338 ± 0.0001 c
Laminaria and chitin	0.1499 ± 0.0033 e
Laminaria	0.2258 ± 0.0075 d
V 5 hyphal wall and chitin	0.3866 ± 0.0001 b
V 5 hyphal wall	0.6747 ± 0.0011 a
Chitin	0.0979 ± 0.0054 f
No inducer	0.3433 ± 0.0033 c

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.9: β-glucanase activity of T 9 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.7112 ± 0.0071 b*
V 5 hyphal wall and *Laminaria*	0.1314 ± 0.0001 c
Laminaria and chitin	0.0831 ± 0.0002 e
Laminaria	0.0961 ± 0.0018 d
V 5 hyphal wall and chitin	0.1261 ± 0.0011 c
V 5 hyphal wall	0.7853 ± 0.0011 a
Chitin	0.1032 ± 0.0128 d
No inducer	0.1066 ± 0.0025 d

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.10: β-glucanase activity of T 2 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.3499 ± 0.0016 d*
V 5 hyphal wall and *Laminaria*	0.3899 ± 0.0016 cd
Laminaria and chitin	0.4466 ± 0.0033 bc
Laminaria	0.4216 ± 0.0051 c
V 5 hyphal wall and chitin	0.7685 ± 0.0028 a
V 5 hyphal wall	0.4533 ± 0.0017 bc
Chitin	0.4491 ± 0.0141 bc
No inducer	0.4908 ± 0.0525 b

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.11: β-glucanase activity of T 6 (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.3652 ± 0.0423 b*
V 5 hyphal wall and *Laminaria*	0.2123 ± 0.0043 c
Laminaria and chitin	0.1185 ± 0.0005 de
Laminaria	0.1583 ± 0.0243 cd
V 5 hyphal wall and chitin	0.5982 ± 0.0085 a
V 5 hyphal wall	0.1125 ± 0.0055 de
Chitin	0.1237 ± 0.0037 de
No inducer	0.0989 ± 0.0072 e

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.12: β-glucanase activity of T x (4-day culture)

Inducer	μ moles glucose / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.4729 ± 0.0112 a*
V 5 hyphal wall and *Laminaria*	0.3833 ± 0.0017 b
Laminaria and chitin	0.3201 ± 0.0051 c
Laminaria	0.3633 ± 0.0133 b
V 5 hyphal wall and chitin	0.0921 ± 0.0004 e
V 5 hyphal wall	0.0961 ± 0.0002 e
Chitin	0.0949 ± 0.0008 e
No inducer	0.1895 ± 0.0004 d

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.13: Responses of five Trichoderma strains to different inducer combinations (V:V 5 hyphal wall; C:Chitin; L:Laminaria meal) in producing β-glucanase (μ moles glucose/min/mg protein)

T 1	(V)	(V+C)	None	(V+L)	(L)	(L+V+C)	(L+C)	(C)
	0.674	0.386	0.343	0.333	0.225	0.162	0.149	0.097
T 9	(V)	(L+V+C)	(V+L)	(V+C)	None	(C)	(L)	(L+C)
	0.785	0.711	0.131	0.126	0.106	0.103	0.096	0.083
T 2	(V+C)	None	(V)	(C)	(L+C)	(L)	(V+L)	(L+V+C)
	0.768	0.490	0.453	0.449	0.446	0.421	0.389	0.349
T 6	(V+C)	(L+V+C)	(V+L)	(L)	(C)	(L+C)	(V)	None
	0.598	0.365	0.212	0.158	0.123	0.118	0.112	0.098
T X	(L+V+C)	(V+L)	(L)	(L+C)	None	(V)	(C)	(V+C)
	0.472	0.383	0.363	0.320	0.189	0.096	0.094	0.092

* Means within underlined were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.14: Chitinase activity of samples from T 1 (4-day culture)

Inducer	μ moles N-acetylglucosamine / min / mg protein
V 5 hyphal wall, Laminaria and chitin	0.0029 ± 0.0005 c*
V 5 hyphal wall and Laminaria	0.1016 ± 0.0483 ab
Laminaria and chitin	0.0167 ± 0.0048 c
Laminaria	0.0143 ± 0.0013 c
V 5 hyphal wall and chitin	0.0345 ± 0.0071 bc
V 5 hyphal wall	0.1170 ± 0.0001 a
Chitin	0.0991 ± 0.0408 ab
No inducer	0.0768 ± 0.0854 abc

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.15: Chitinase activity of samples from T 9 (4-day culture)

Inducer	μ moles N-acetylglucosamine / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0379 ± 0.0026 bc*
V 5 hyphal wall and *Laminaria*	0.0266 ± 0.0036 bcd
Laminaria and chitin	0.0111 ± 0.0025 cd
Laminaria	0.0029 ± 0.0003 d
V 5 hyphal wall and chitin	0.0139 ± 0.0006 cd
V 5 hyphal wall	0.1983 ± 0.0251 a
Chitin	0.0460 ± 0.0006 b
No inducer	0.0168 ± 0.0068 bcd

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.16: Chitinase activity of samples from T 2 (4-day culture)

Inducer	μ moles N-acetylglucosamine / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0454 ± 0.0129 bc*
V 5 hyphal wall and *Laminaria*	0.0451 ± 0.0035 c
Laminaria and chitin	0.1241 ± 0.0258 a
Laminaria	0.0533 ± 0.0301 bc
V 5 hyphal wall and chitin	0.1071 ± 0.0001 ab
V 5 hyphal wall	0.0604 ± 0.0004 bc
Chitin	0.0415 ± 0.0061 c
No inducer	0.0556 ± 0.0081 bc

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.17: Chitinase activity of samples from T 6 (4-day culture)

Inducer	μ moles N-acetylglucosamine / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.1708 ± 0.0025 b*
V 5 hyphal wall and *Laminaria*	0.1799 ± 0.0116 b
Laminaria and chitin	0.0299 ± 0.0133 de
Laminaria	0.0608 ± 0.0075 c
V 5 hyphal wall and chitin	0.5555 ± 0.0001 a
V 5 hyphal wall	0.0158 ± 0.0092 e
Chitin	0.0501 ± 0.0001 cd
No inducer	0.0275 ± 0.0025 de

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.18: Chitinase activity of samples from T x (4-day culture)

Inducer	μ moles N-acetylglucosamine / min / mg protein
V 5 hyphal wall, *Laminaria* and chitin	0.0247 ± 0.0011 b*
V 5 hyphal wall and *Laminaria*	0.0562 ± 0.0042 ab
Laminaria and chitin	0.1073 ± 0.0044 a
Laminaria	0.0372 ± 0.0127 b
V 5 hyphal wall and chitin	0.0585 ± 0.0085 ab
V 5 hyphal wall	0.0329 ± 0.0038 b
Chitin	0.1021 ± 0.0021 a
No inducer	0.0609 ± 0.0201 ab

* Means with the same letter were not significantly different, p=0.05, according to Duncan's multiple range test.

Table 3.19: Responses of five *Trichoderma* strains to different inducer combinations (V:V 5 hyphal wall; C:Chitin; L:*Laminaria* meal) in producing Chitinase (μ moles N-acetylglucosamine/min /mg protein)

T1	(V)	(V+L)	(C)	None	(V+C)	(L+C)	(L)	(L+V+C)
	0.117	0.101	0.099	0.076	0.034	0.016	0.014	0.002
T9	(V)	(C)	(L+V+C)	(V+L)	None	(V+C)	(L+C)	(L)
	0.198	0.046	0.037	0.026	0.016	0.013	0.011	0.002
T2	(L+C)	(V+C)	(V)	None	(L)	(L+V+C)	(L+V)	(C)
	0.124	0.107	0.060	0.055	0.053	0.045	0.045	0.041
T6	(V+C)	(L+V)	(L+V+C)	(L)	(C)	(L+C)	None	(V)
	0.555	0.179	0.170	0.060	0.050	0.029	0.027	0.015
TX	(L+C)	(C)	None	(V+C)	(L+C)	(L)	(V)	(L+V+C)
	0.107	0.102	0.060	0.058	0.056	0.037	0.032	0.024

* Means within underlined were not significantly different, p=0.05, according to Duncan's multiple range test.

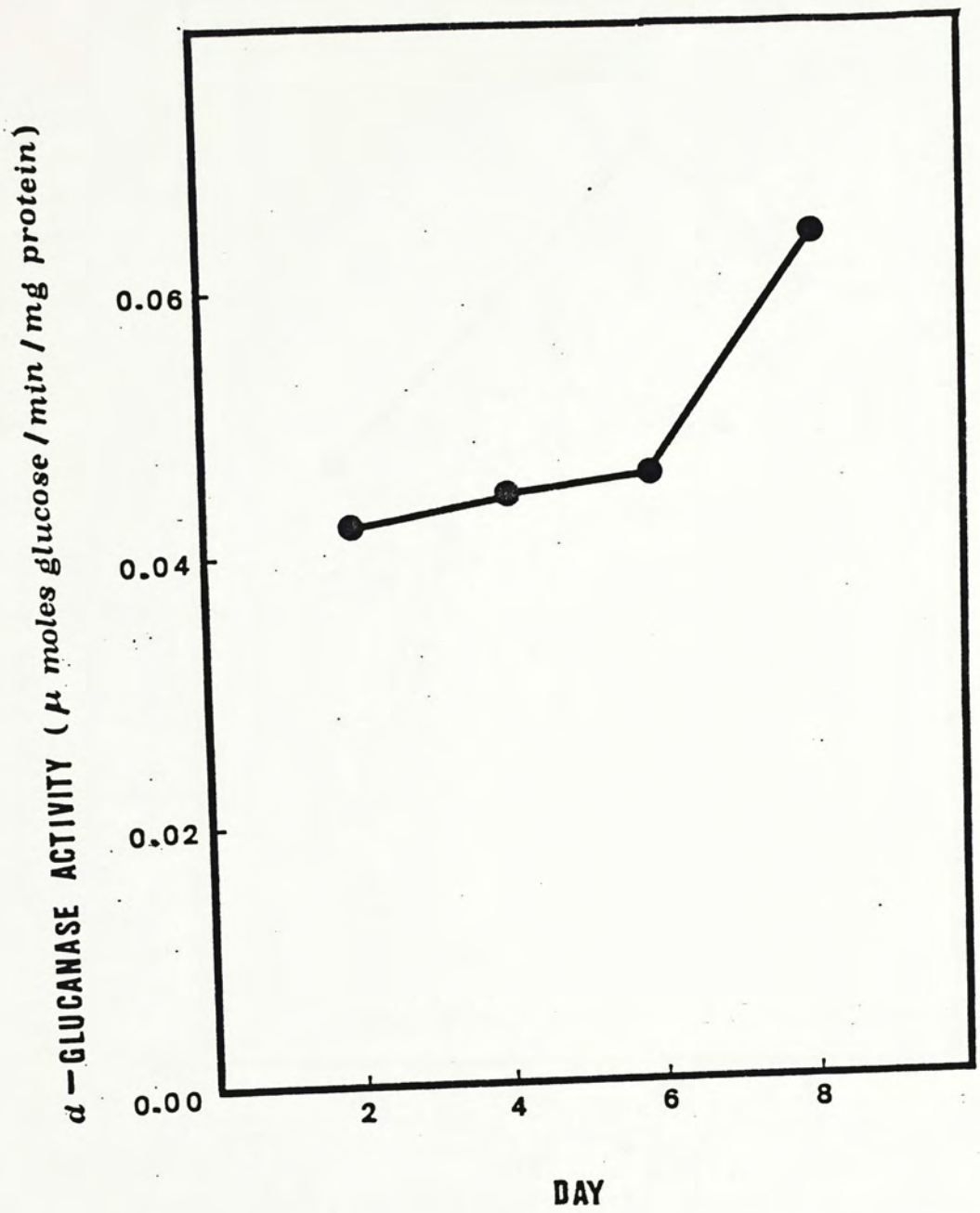


Fig. 3.2. Effect of growth period on d-glucanase production of T 1 with inducers of V 5 hyphal wall and Laminaria

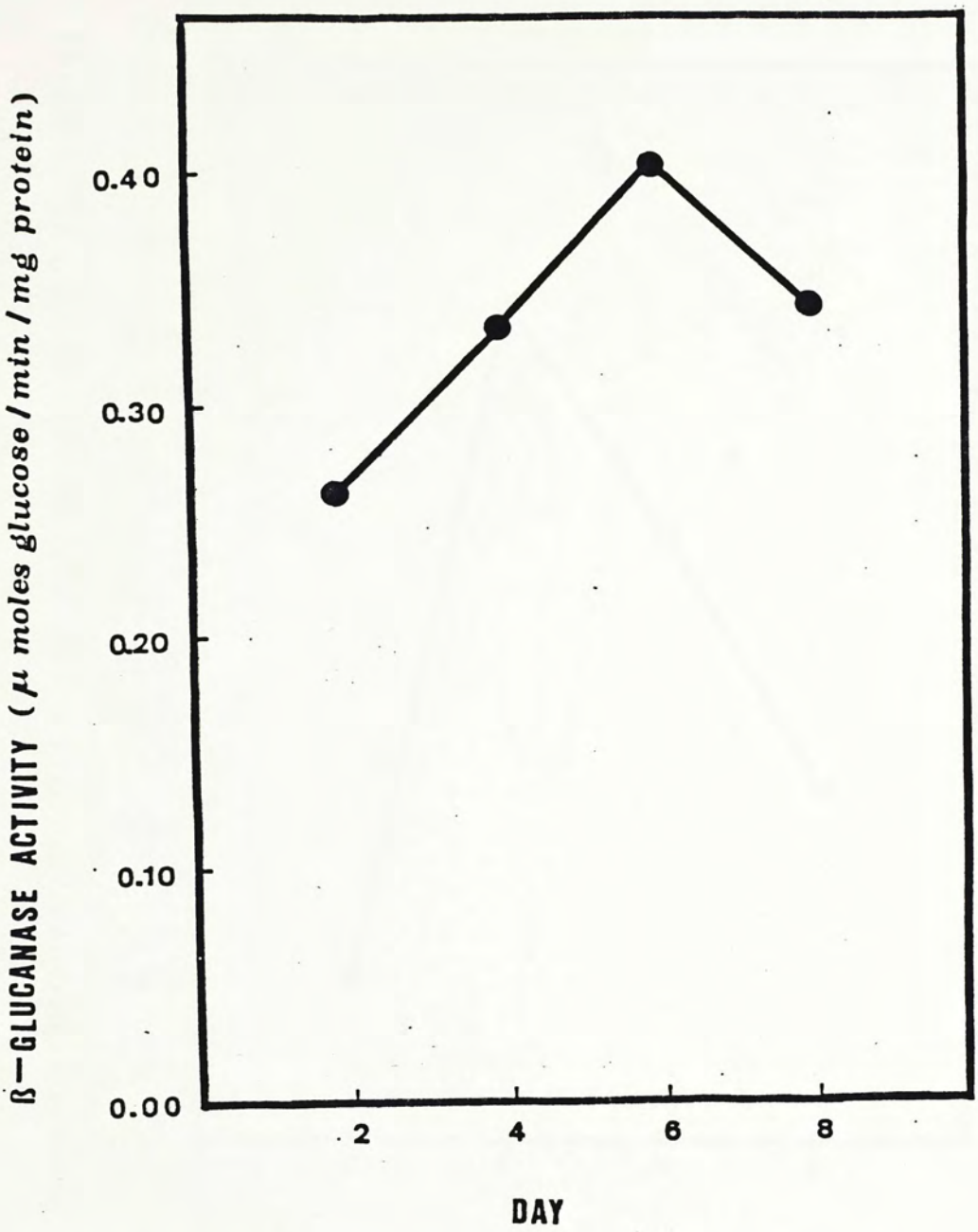


Fig. 3.3. Effect of growth period on ß-glucanase production of T 1 with inducers of V 5 hyphal wall and Laminaria

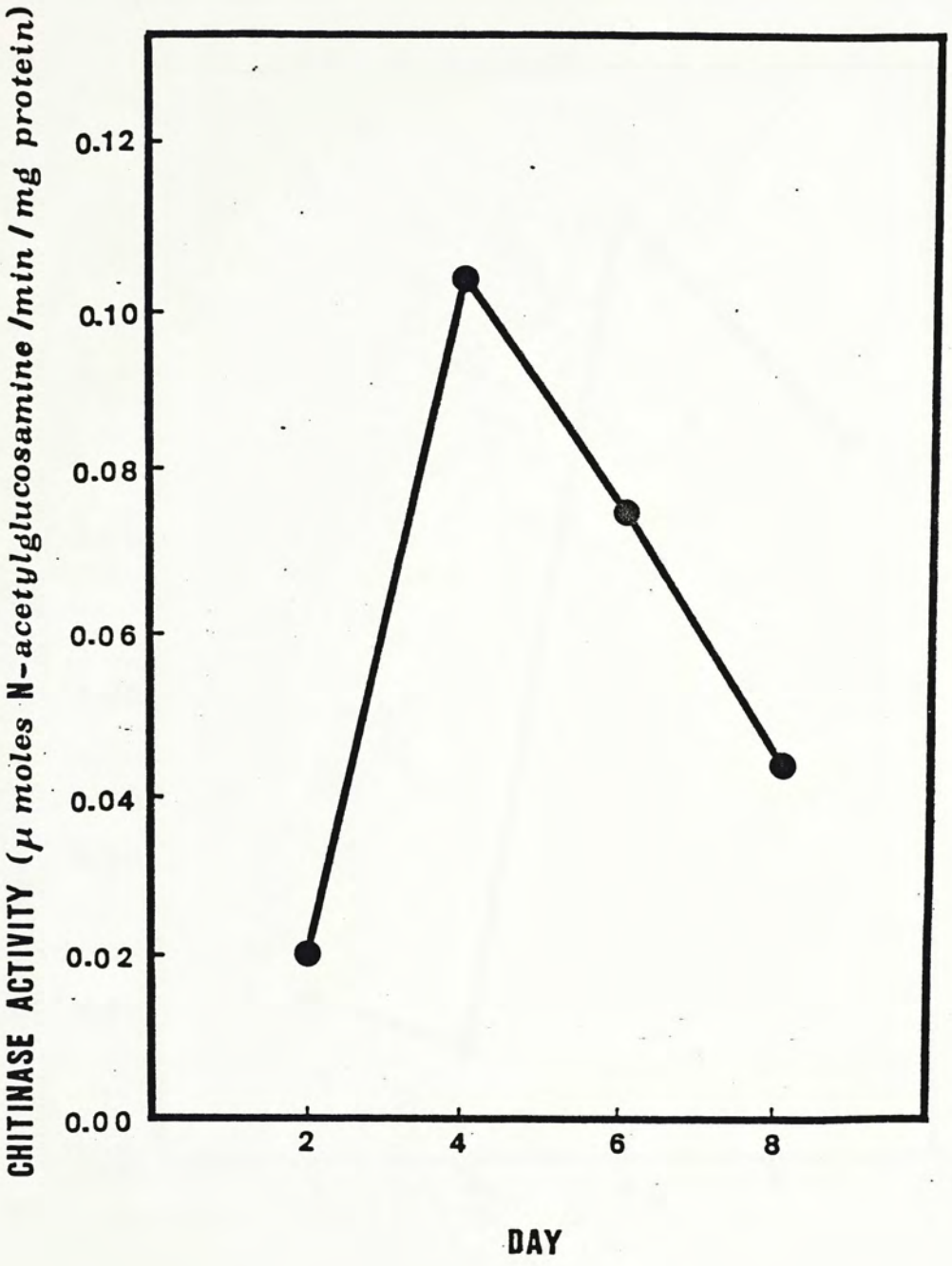


Fig. 3.4. Effect of growth period on chitinase production of T 1 with inducers of V 5 hyphal wall and Laminaria

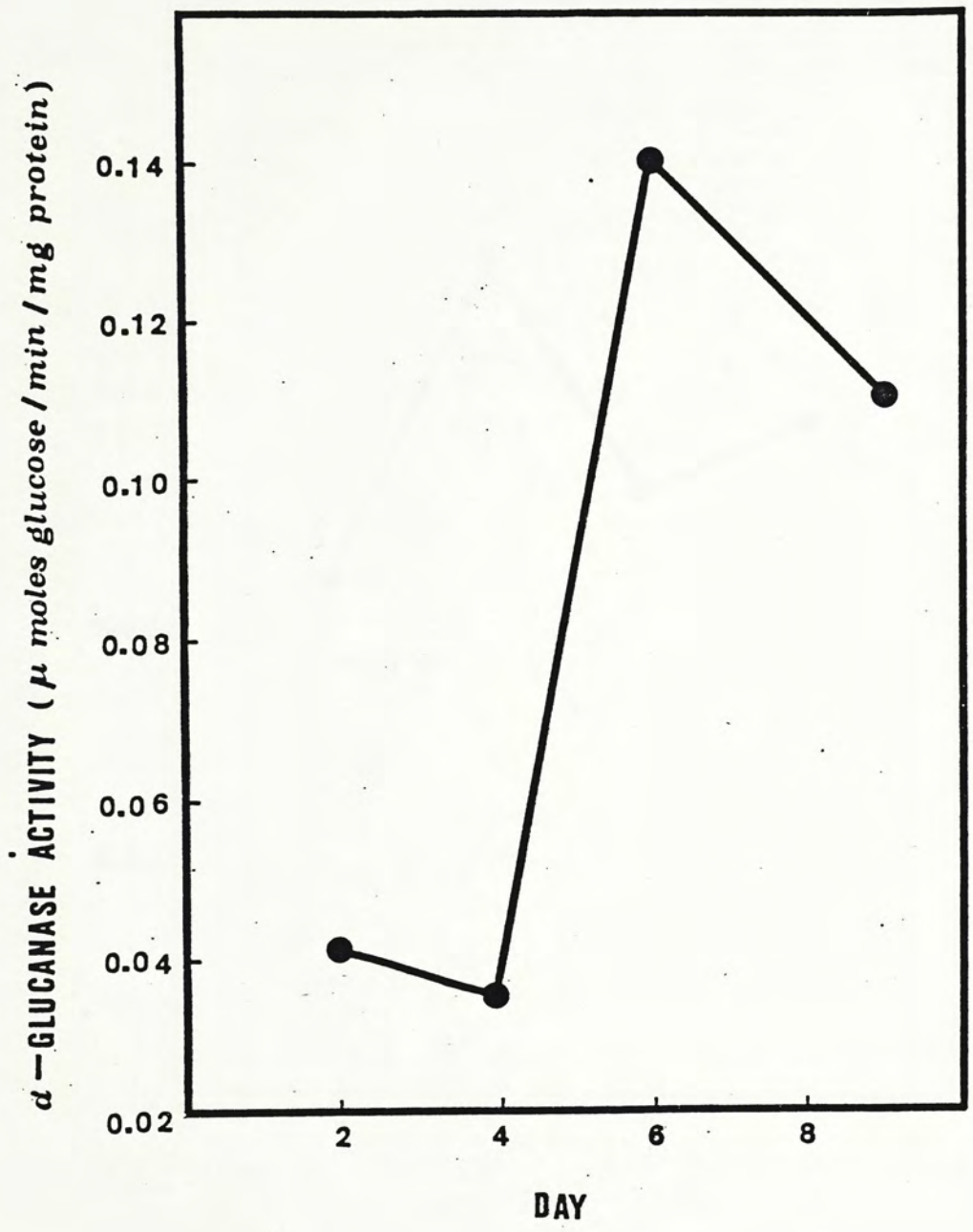


Fig. 3.5. Effect of growth period on *d*-glucanase production of T 9 with inducers of V 5 hyphal wall, Laminaria and chitin

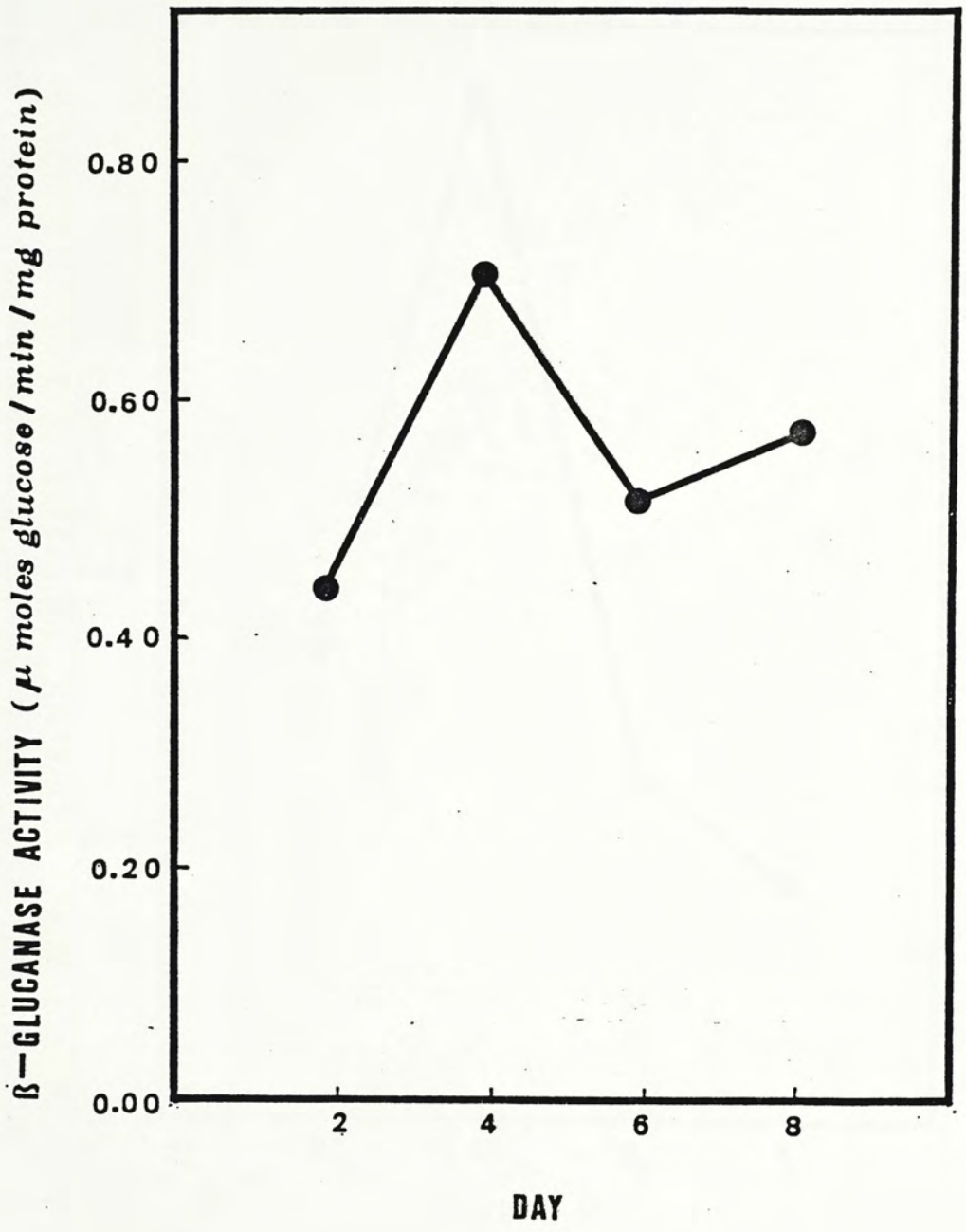


Fig. 3.6. Effect of growth period on ß-glucanase production of T 9 with inducers of V 5 hyphal wall, Laminaria and chitin

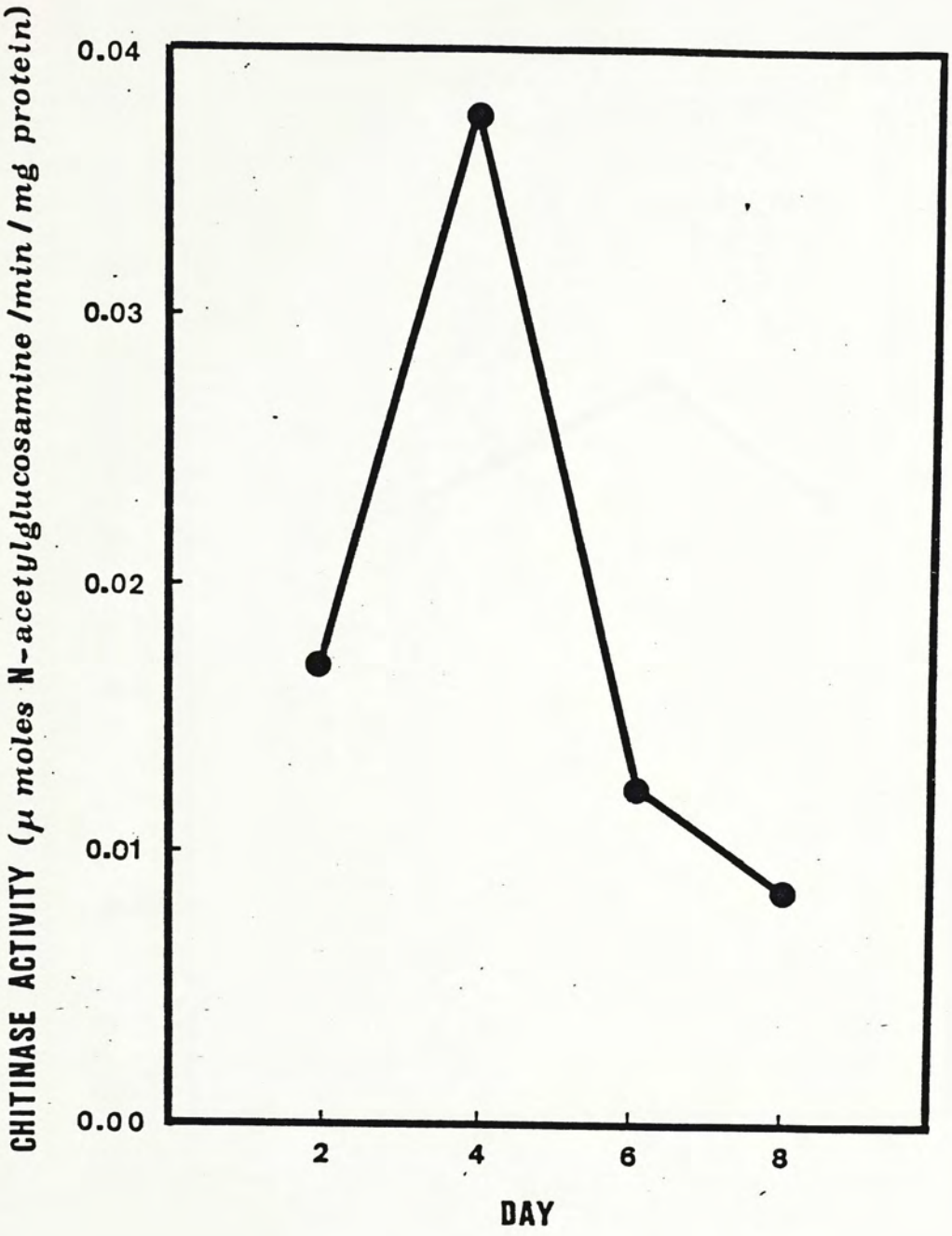


Fig. 3.7. Effect of growth period on chitinase production of T 9 with inducers of V 5 hyphal wall, Laminaria and chitin

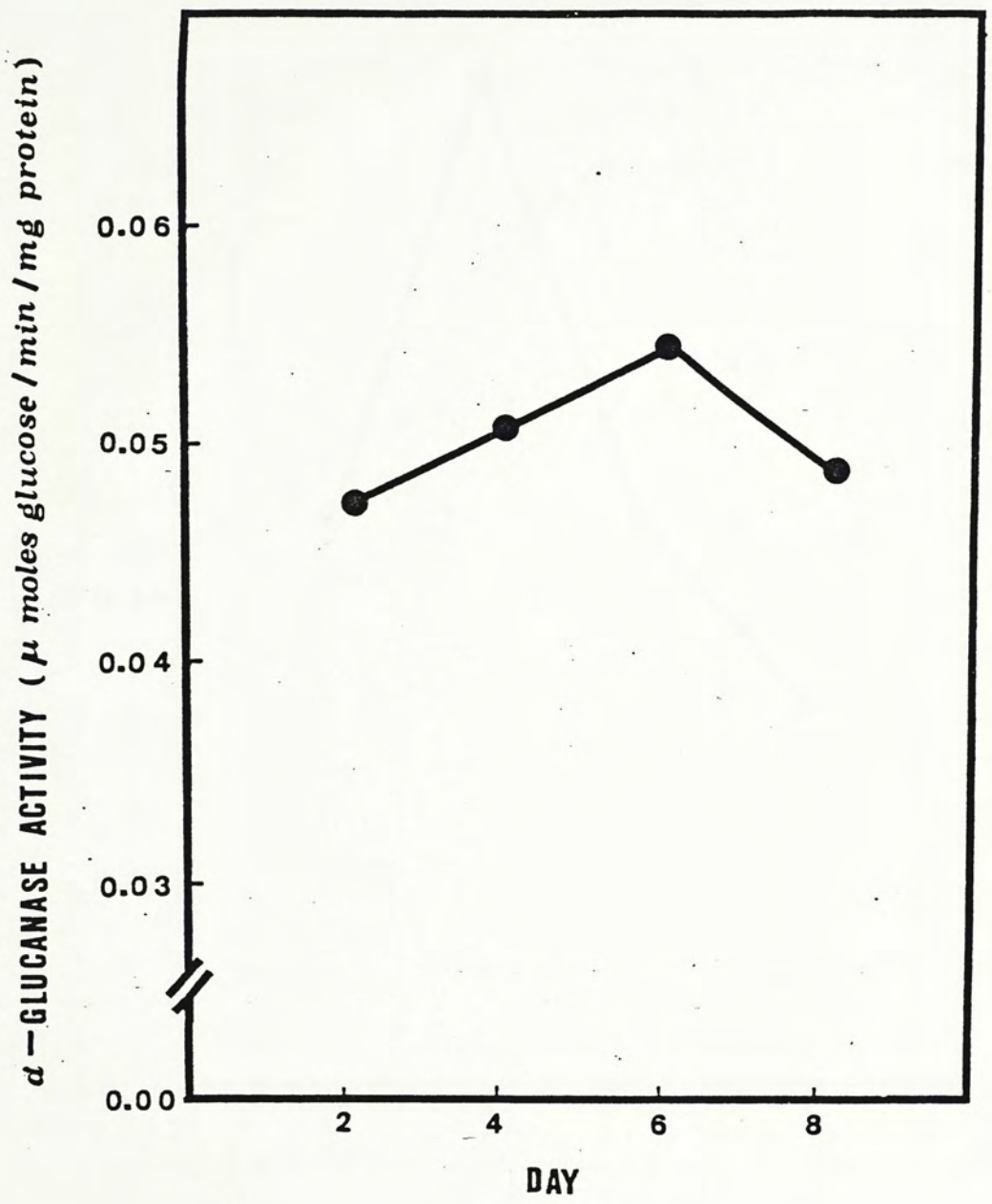


Fig. 3.8. Effect of growth period on *d*-glucanase production of T 2 with inducers of Laminaria and chitin

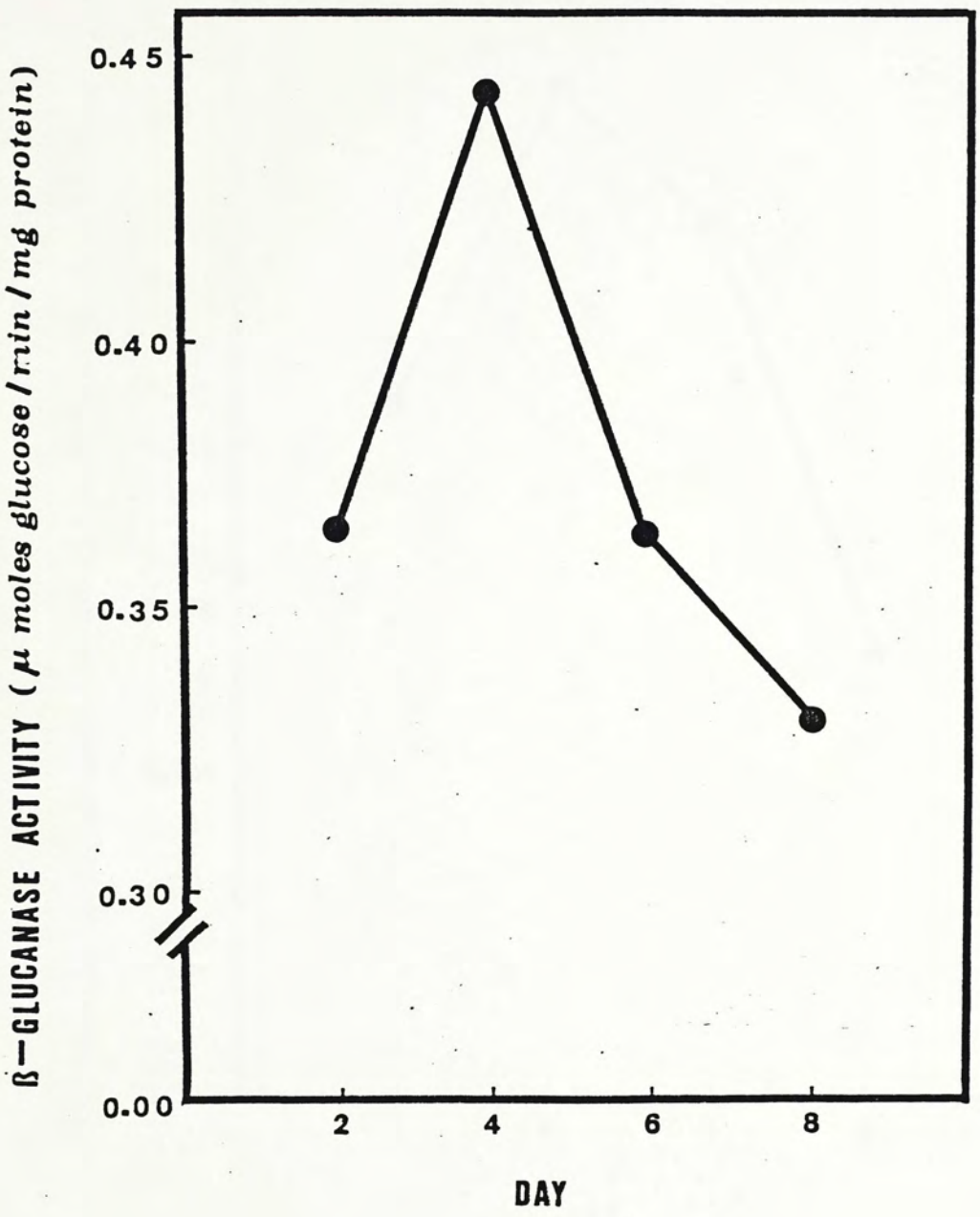


Fig. 3.9. Effect of growth period on ß-glucanase production of T 2 with inducers of Laminaria and chitin

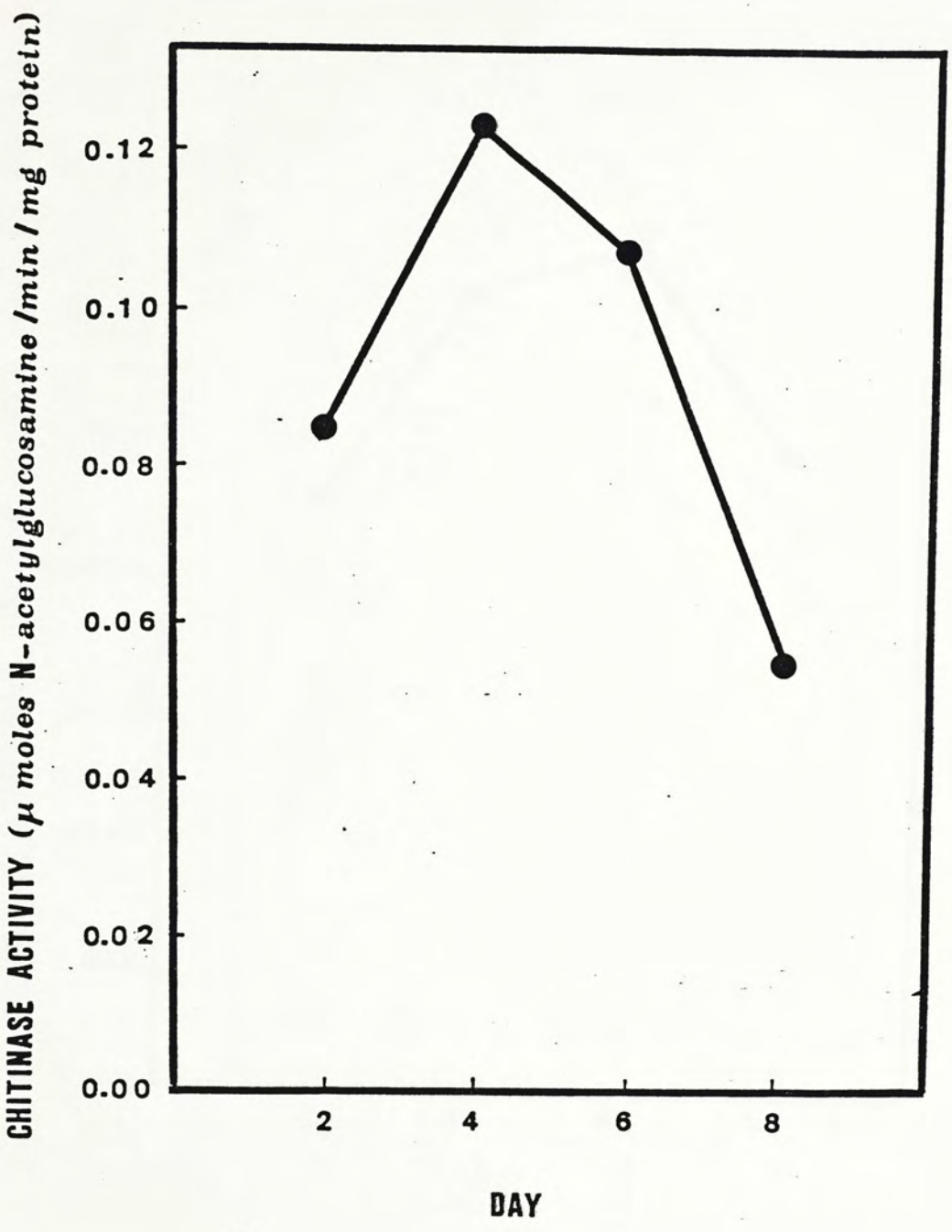


Fig. 3.10. Effect of growth period on chitinase production of T 2 with inducers of Laminaria and chitin

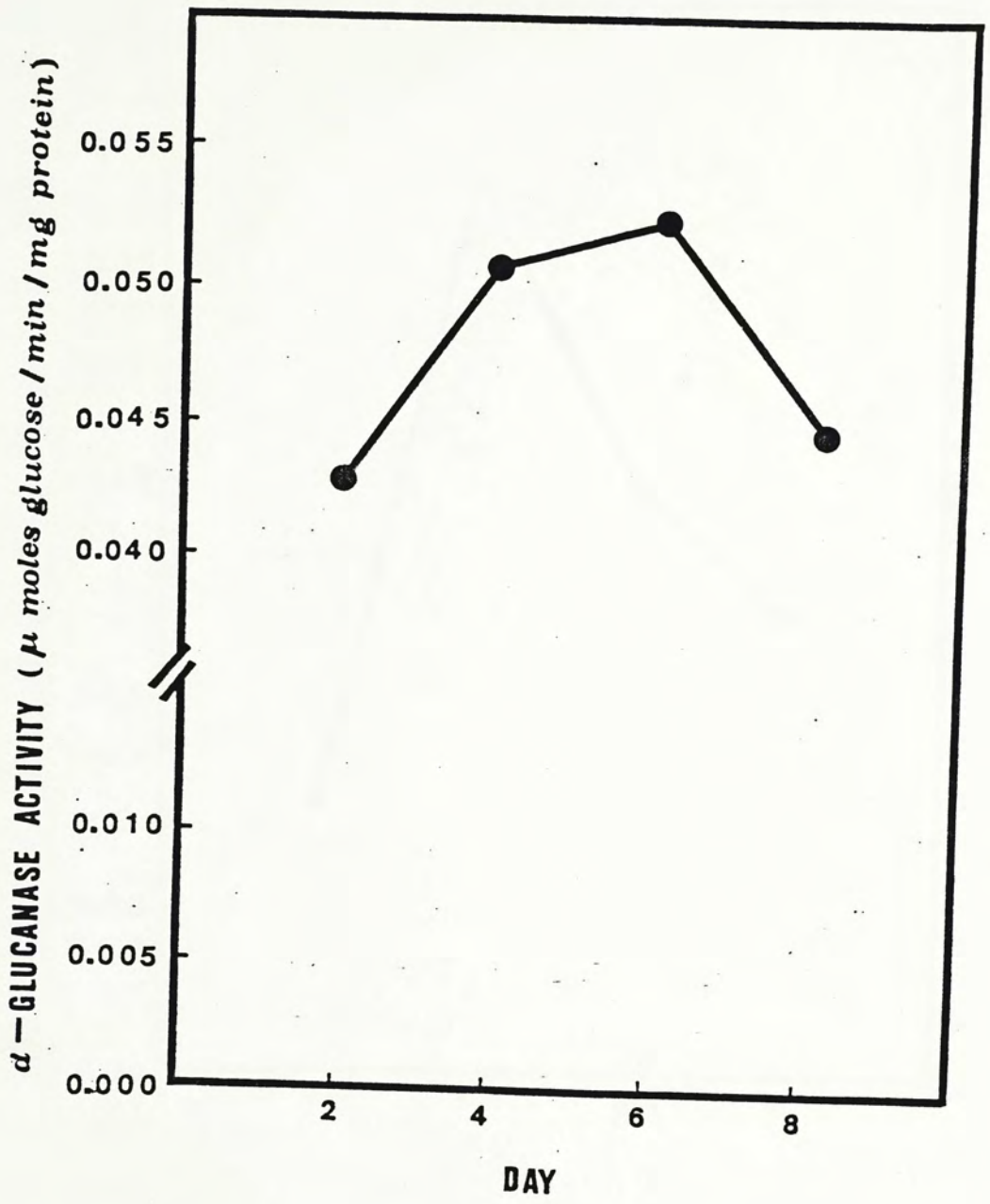


Fig. 3.11. Effect of growth period on α-glucanase production of T 6 with inducers of V 5 hyphal wall, Laminaria and chitin

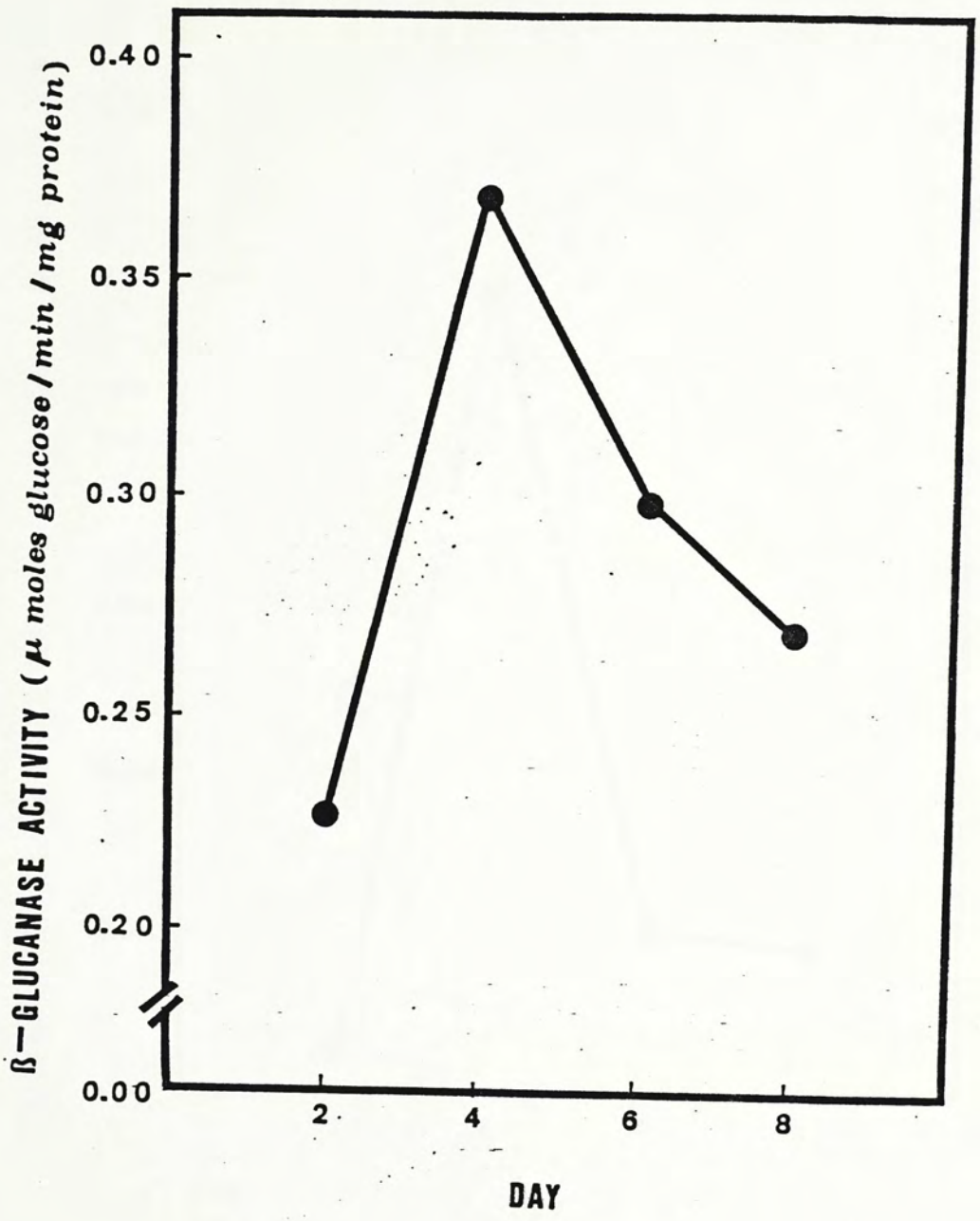


Fig. 3.12. Effect of growth period on ß-glucanase production of T 6 with inducers of V 5 hyphal wall, Laminaria and chitin

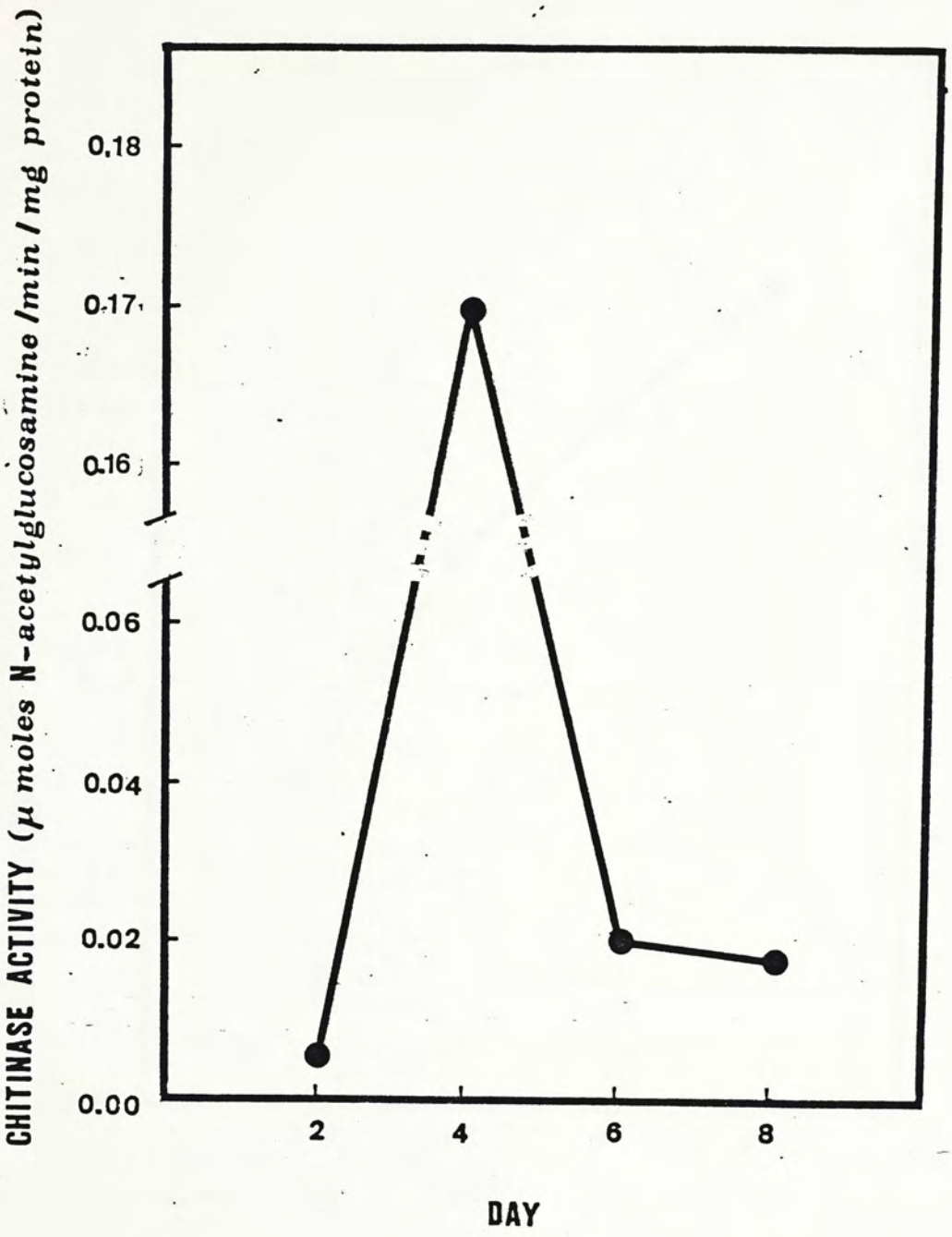


Fig. 3.13. Effect of growth period on chitinase production of T 6 with inducers of V 5 hyphal wall, Laminaria and chitin

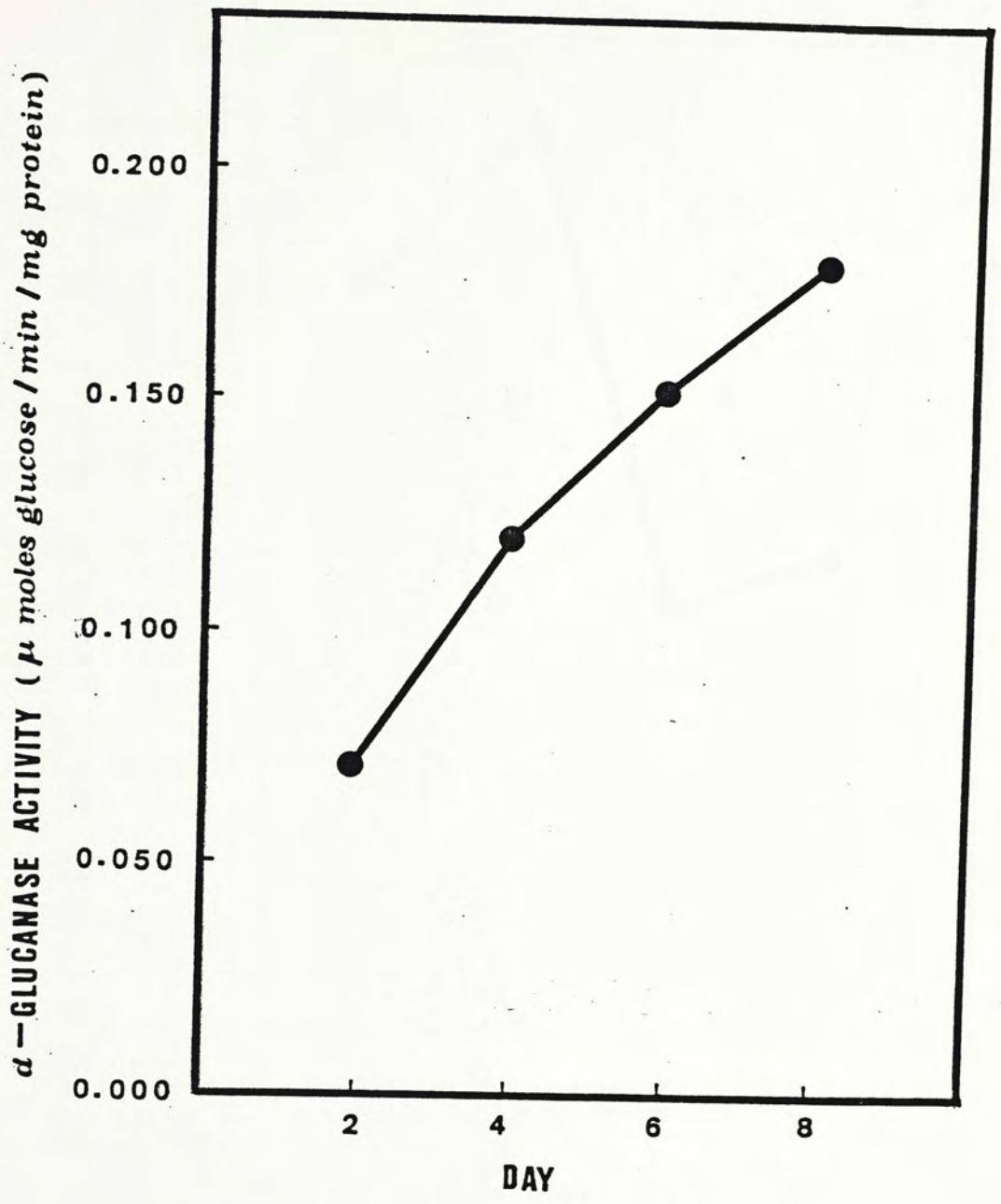


Fig. 3.14. Effect of growth period on d-glucanase production of T X with inducers of Laminaria and chitin

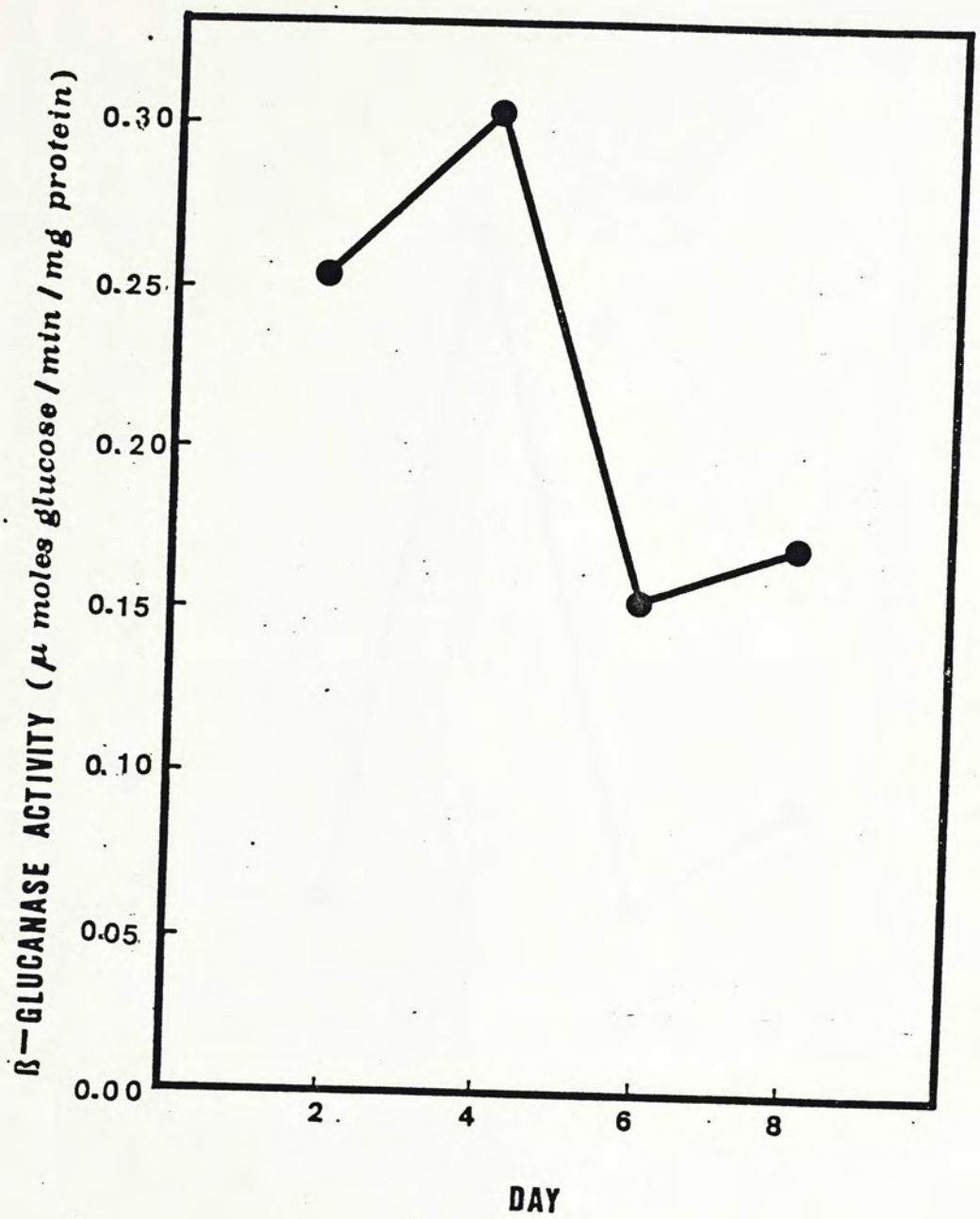


Fig. 3.15. Effect of growth period on ß-glucanase production of T X with inducers of Laminaria and chitin

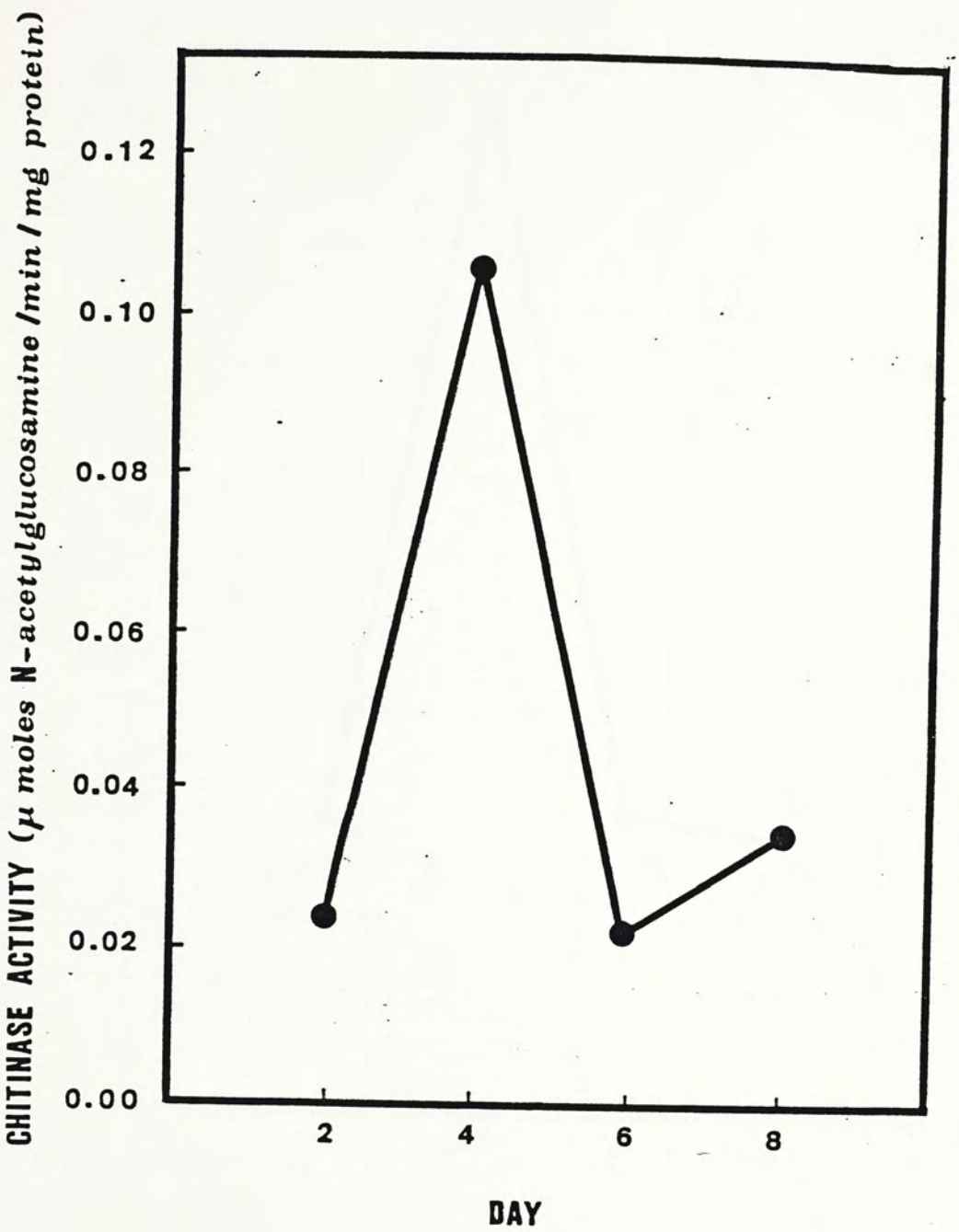


Fig. 3.16. Effect of growth period on chitinase production of T X with inducers of Laminaria and chitin

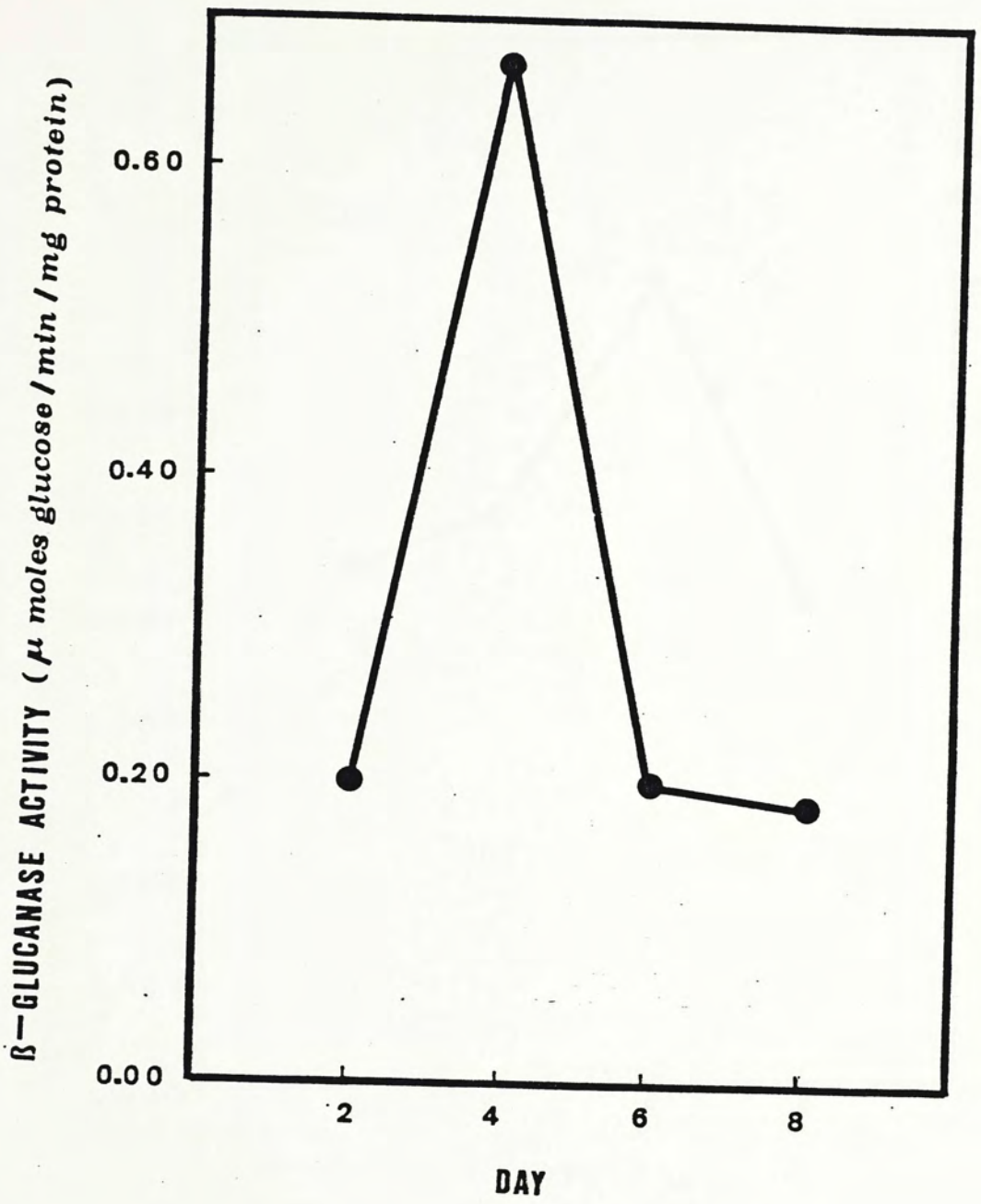


Fig. 3.17. Effect of growth period on ß-glucanase production of T 1 with inducer of V 5 hyphal wall

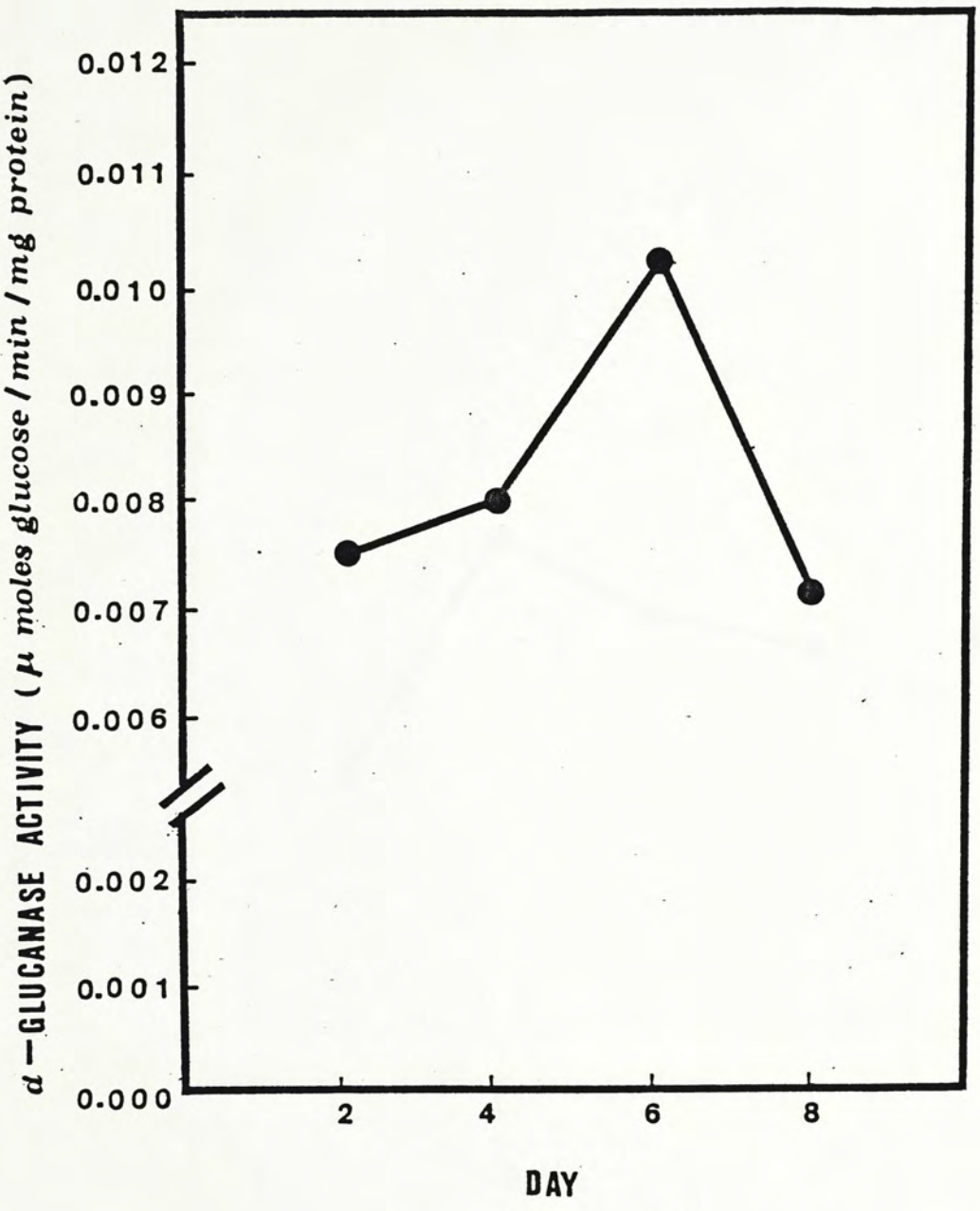


Fig. 3.18. Effect of growth period on α-glucanase production of T 1 with inducer of V 5 hyphal wall

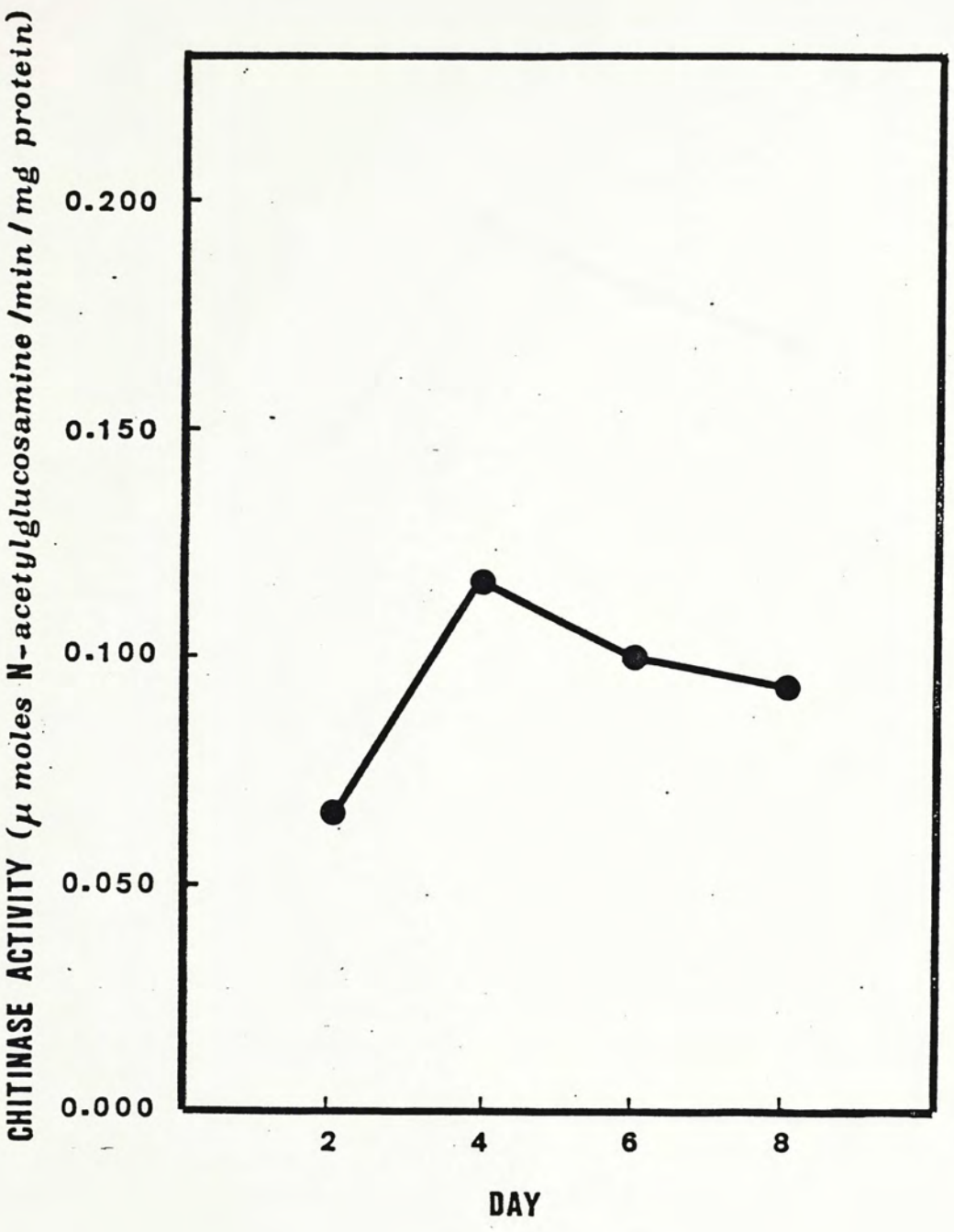


Fig. 3.19. Effect of growth period on chitinase production of T 1 with inducer of V 5 hyphal wall

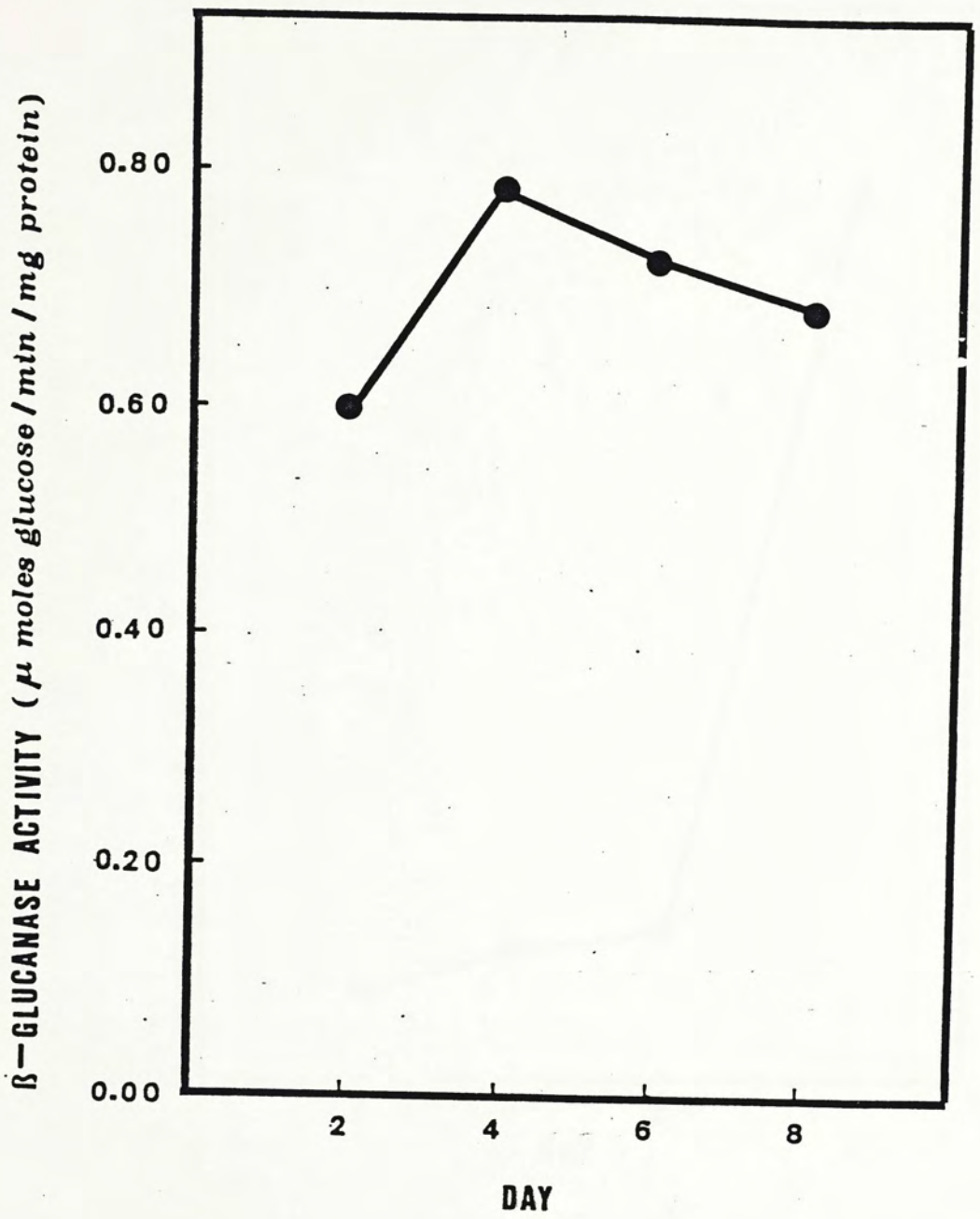


Fig. 3.20: Effect of growth period on ß-glucanase production of T 9 with inducer of V 5 hyphal wall

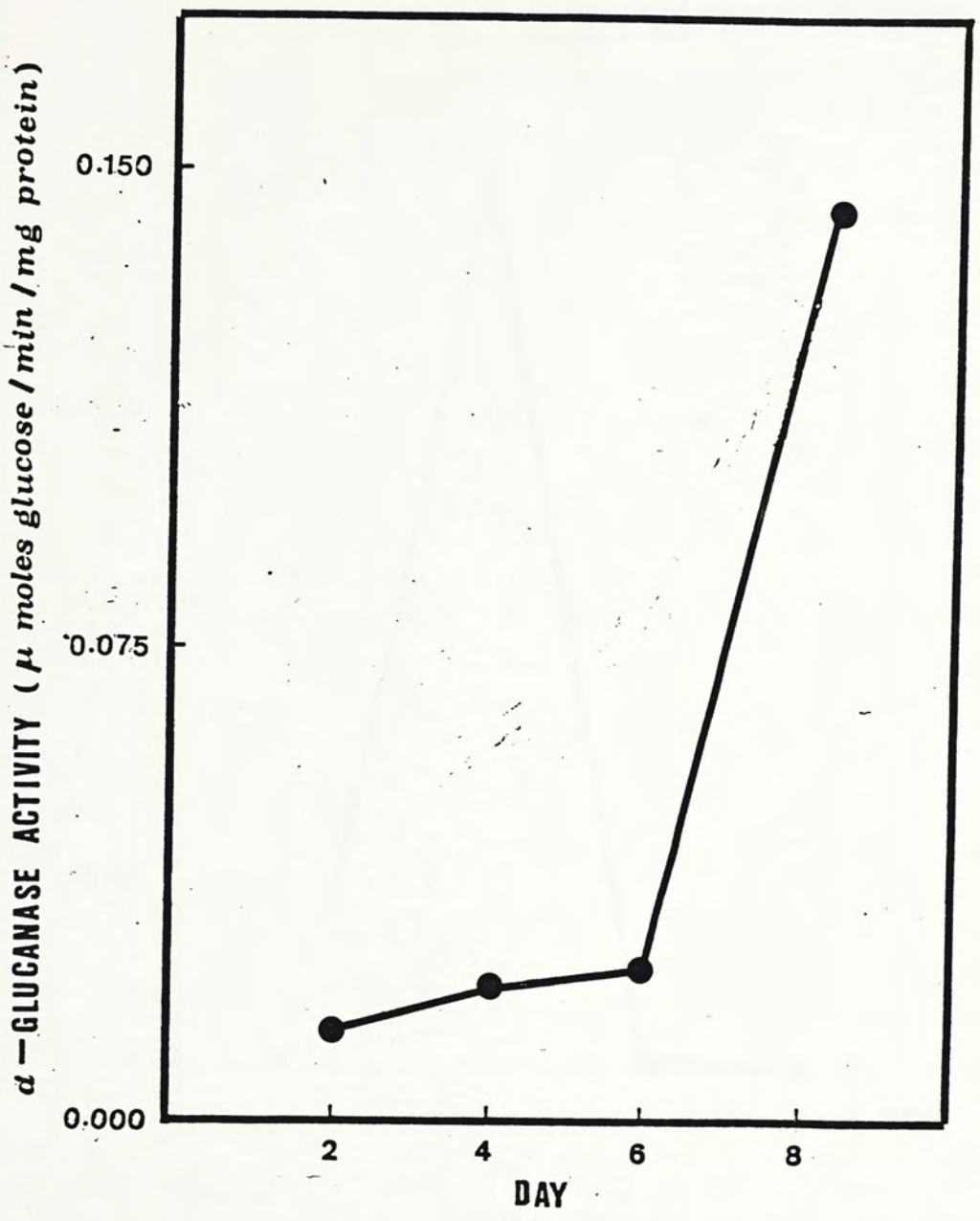


Fig. 3.21. Effect of growth period on d-glucanase production of T 9 with inducer of V 5 hyphal wall

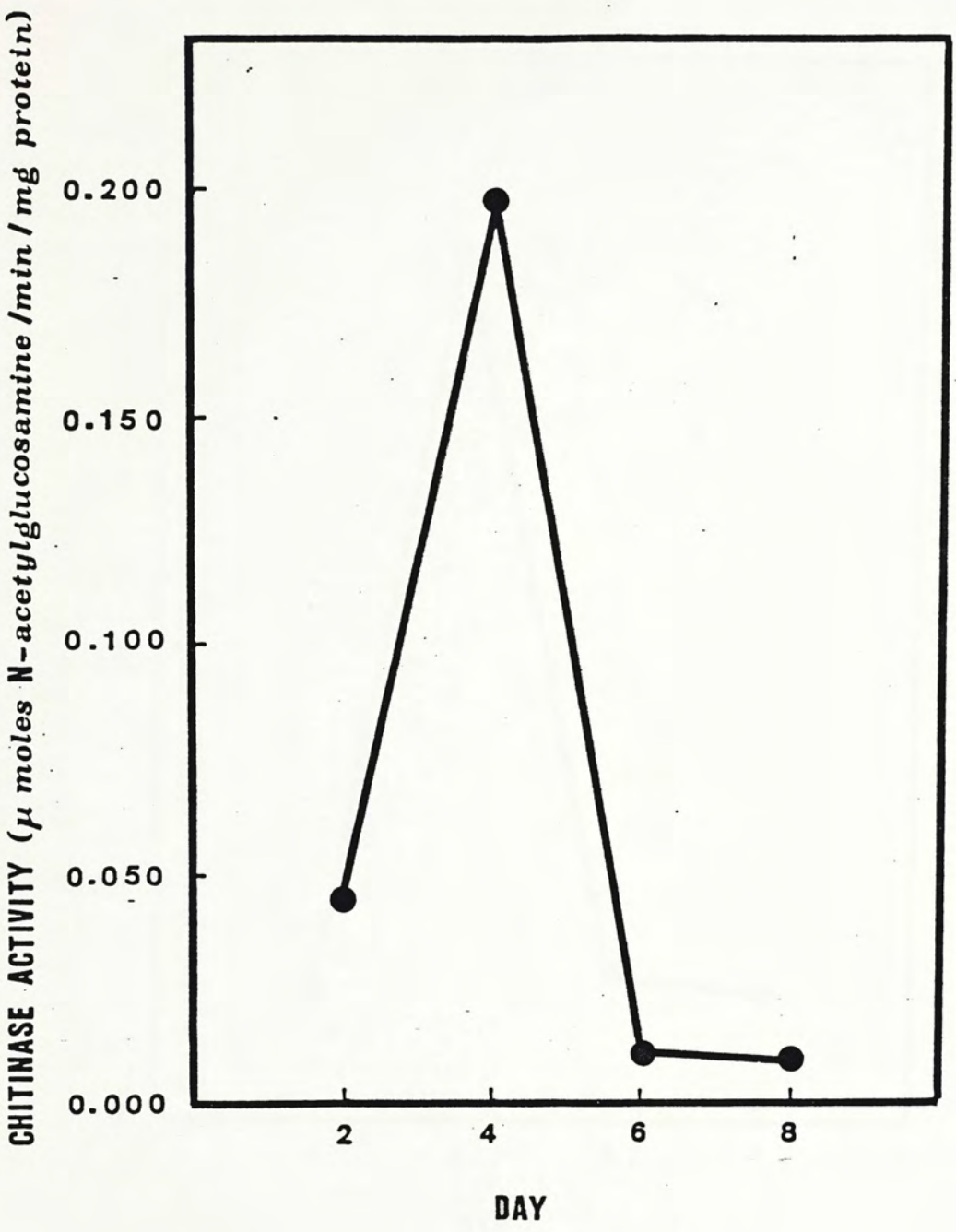


Fig. 3.22: Effect of growth period on chitinase production of T 9 with inducer of V 5 hyphal wall

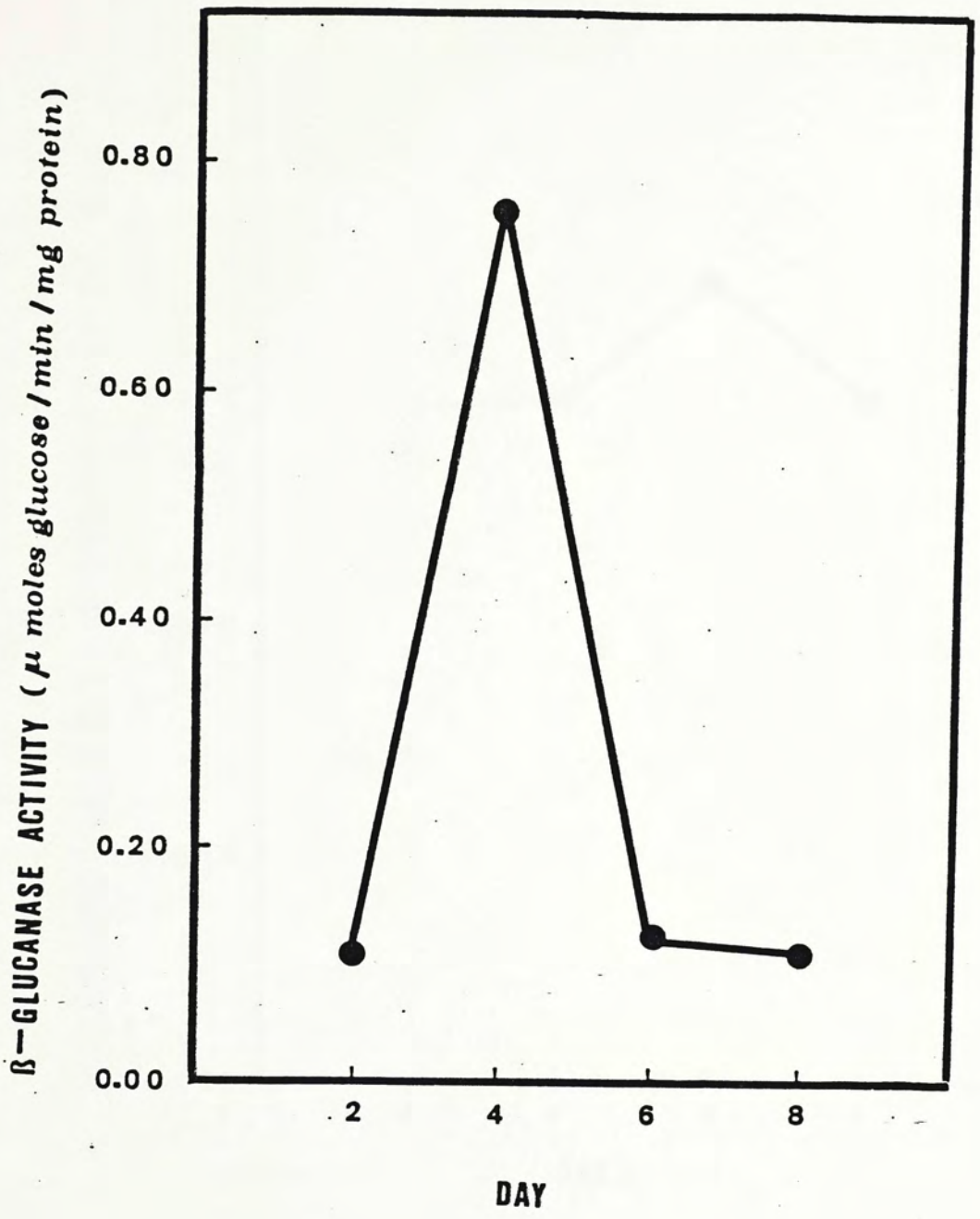


Fig. 3.23. Effect of growth period on ß-glucanase production of T 2 with inducers of V 5 hyphal wall and chitin

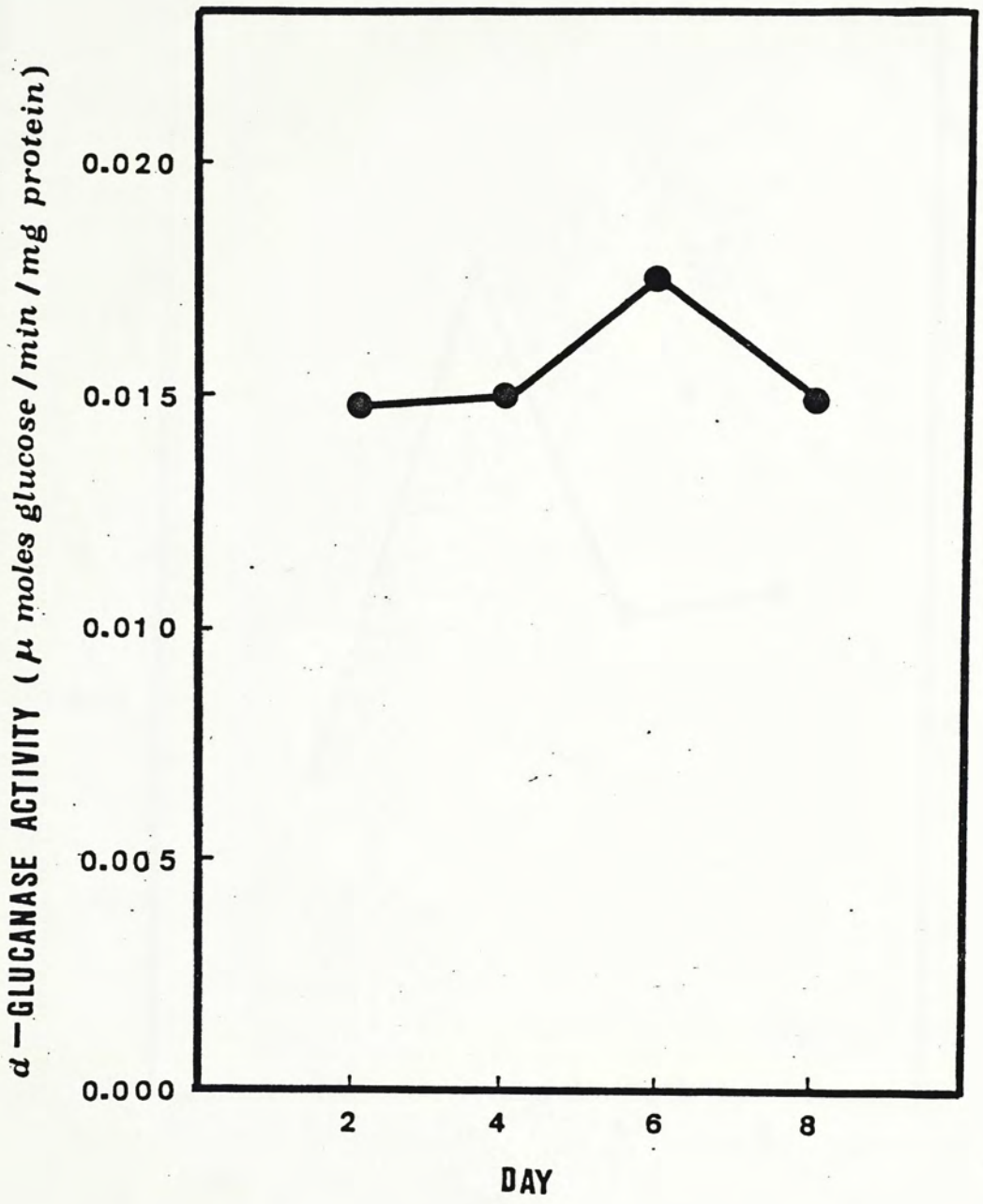


Fig. 3.24. Effect of growth period on α-glucanase production of T 2 with inducers of V 5 hyphal wall and chitin

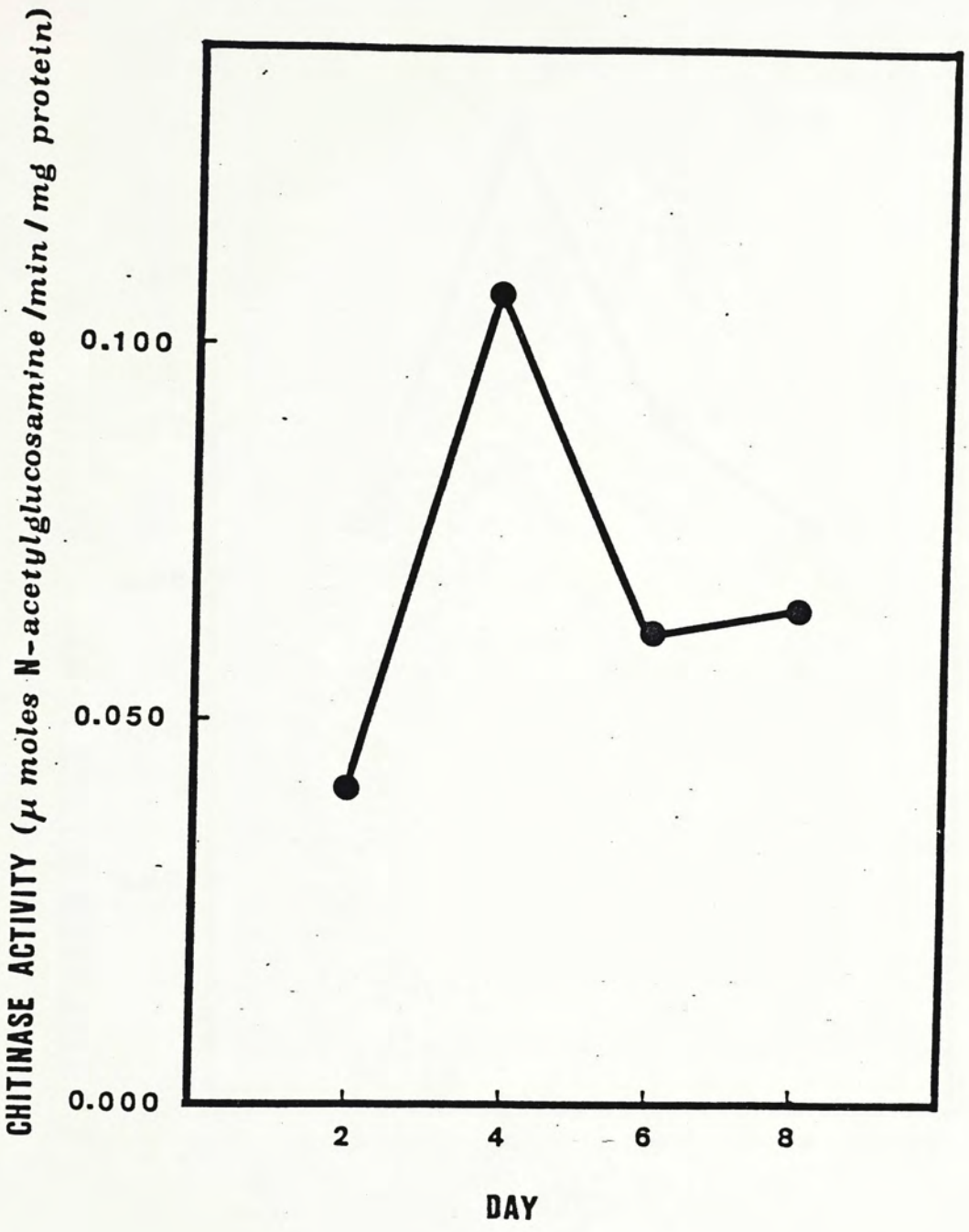


Fig. 3.25. Effect of growth period on chitinase production of T 2 with inducers of V 5 hyphal wall and chitin

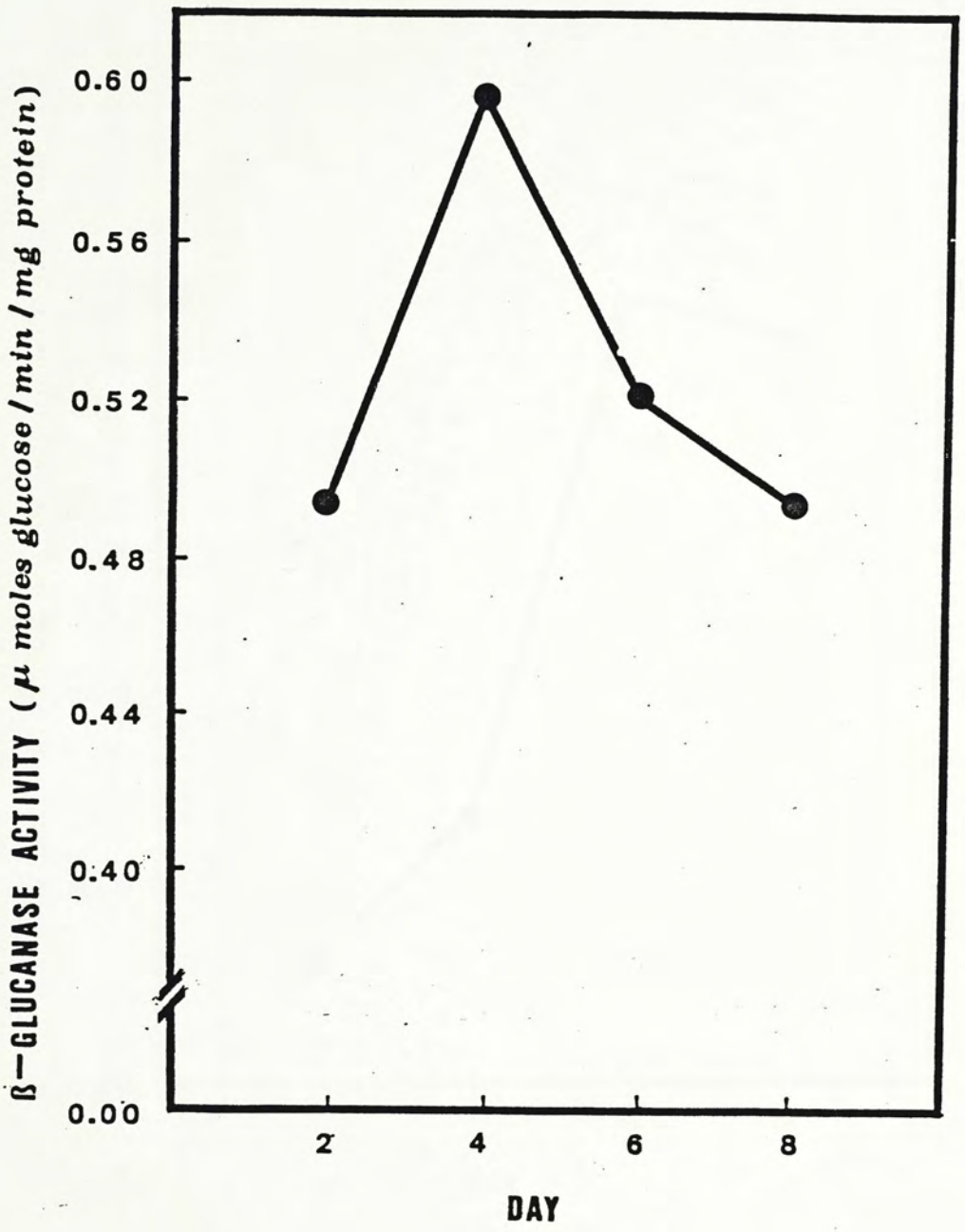


Fig. 3.26. Effect of growth period on ß-glucanase production of T 6 with inducers of V 5 hyphal wall and chitin

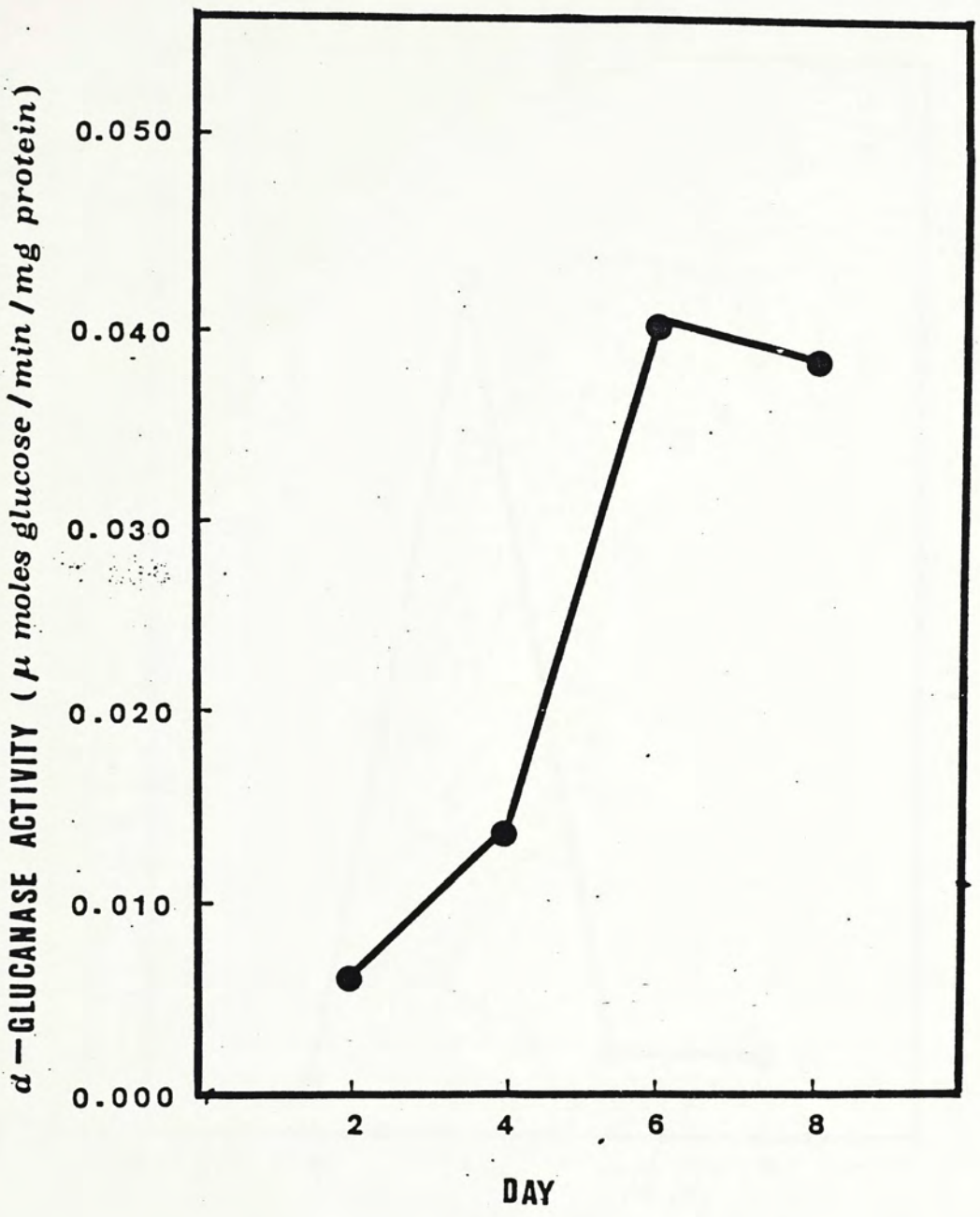


Fig. 3.27. Effect of growth period on α-glucanase production of T 6 with inducers of V-5 hyphal wall and chitin

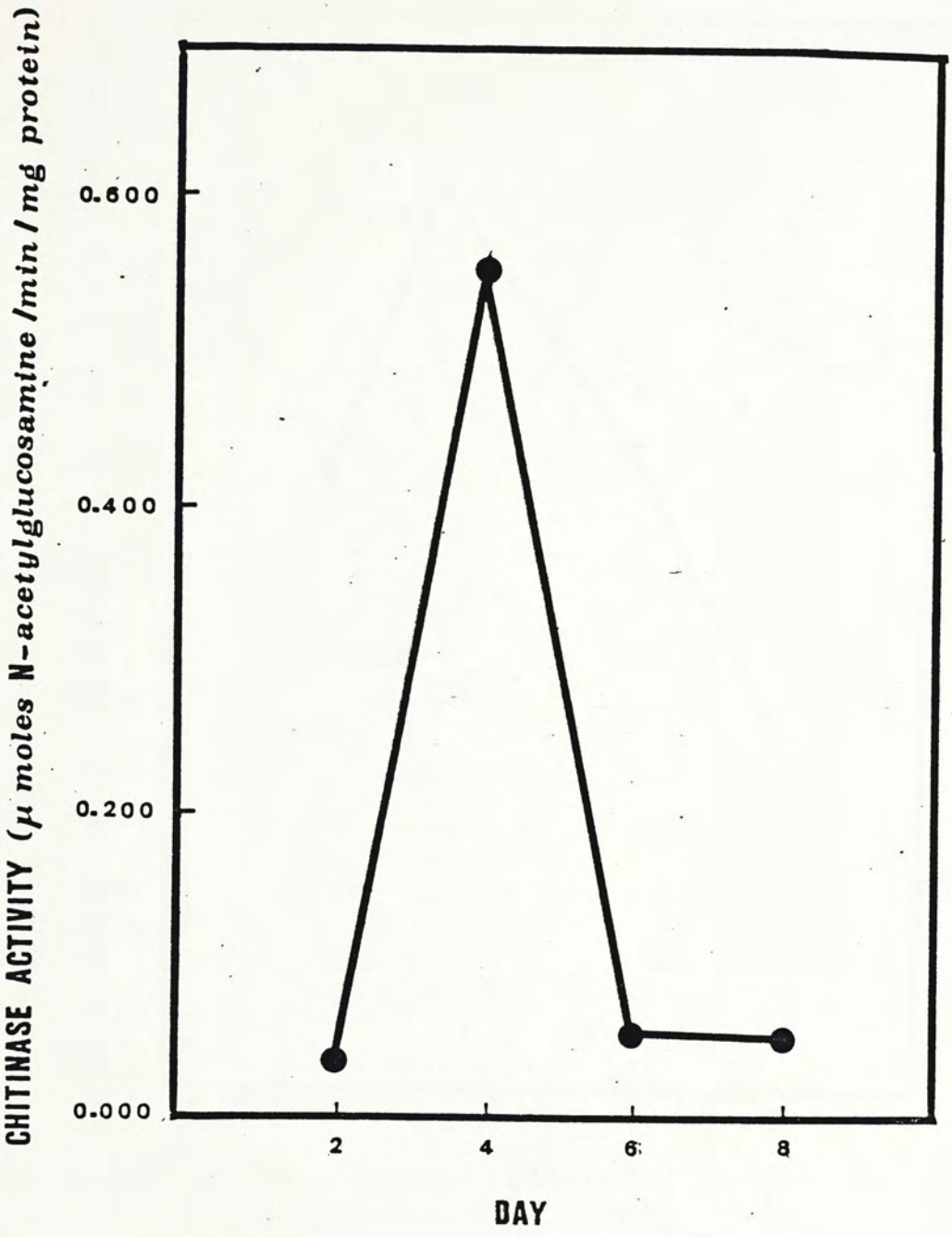


Fig. 3.28. Effect of growth period on chitinase production of T 6 with inducers of V 5 hyphal wall and chitin

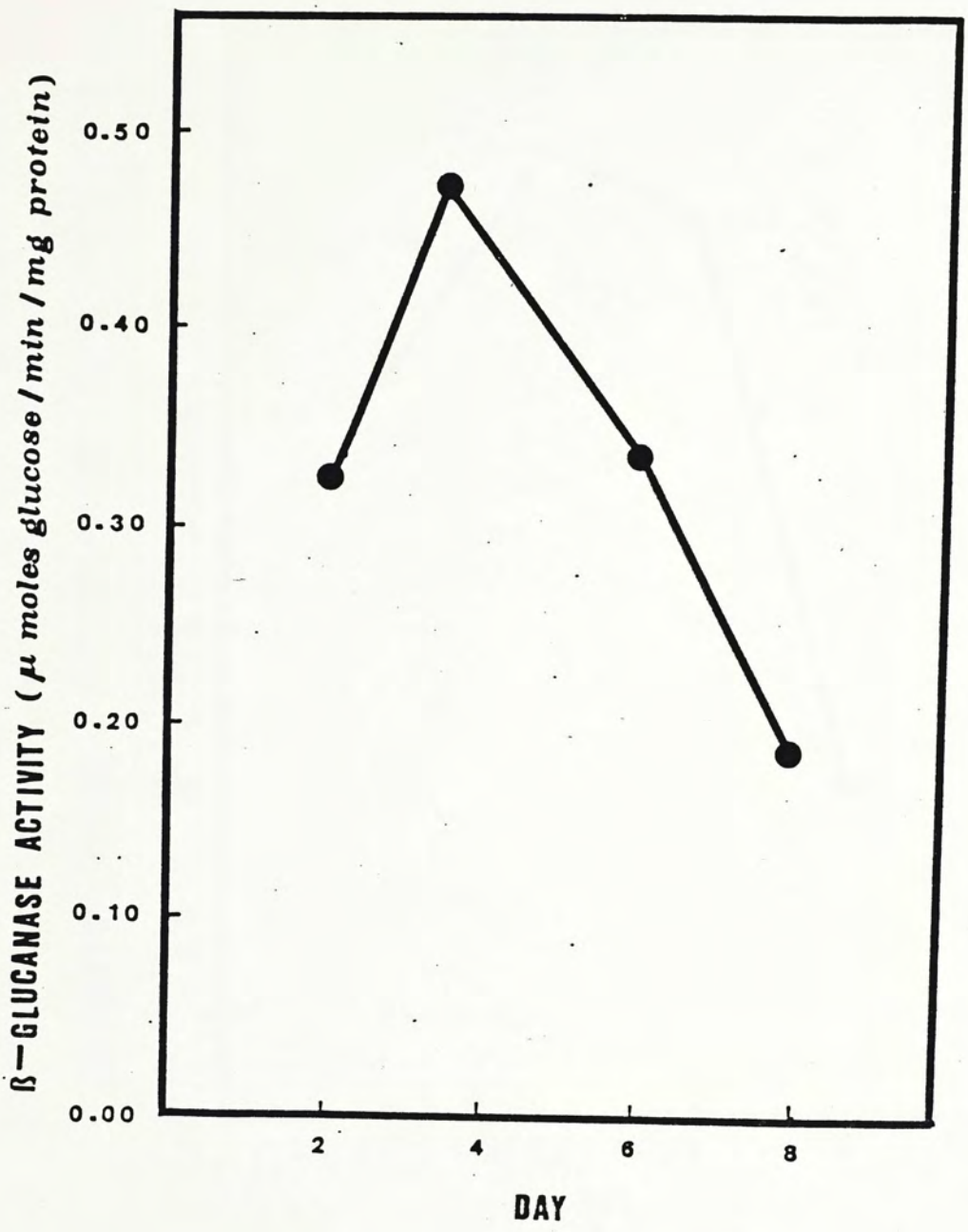


Fig. 3.29. Effect of growth period on ß-glucanase production of T X with inducers of V 5 hyphal wall, Laminaria and chitin

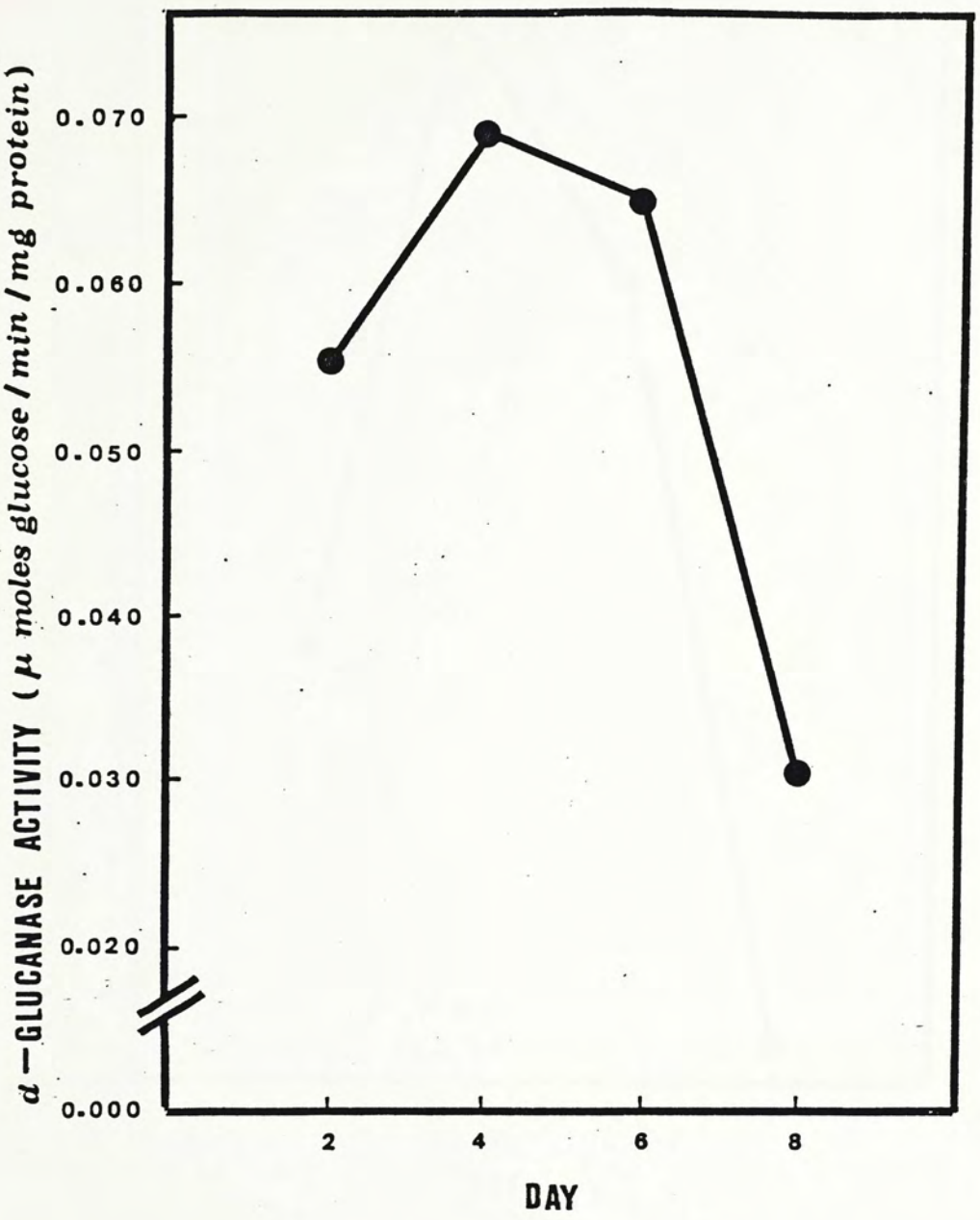


Fig. 3.30. Effect of growth period on d-glucanase production of T X with inducers of V 5 hyphal wall, Laminaria and chitin

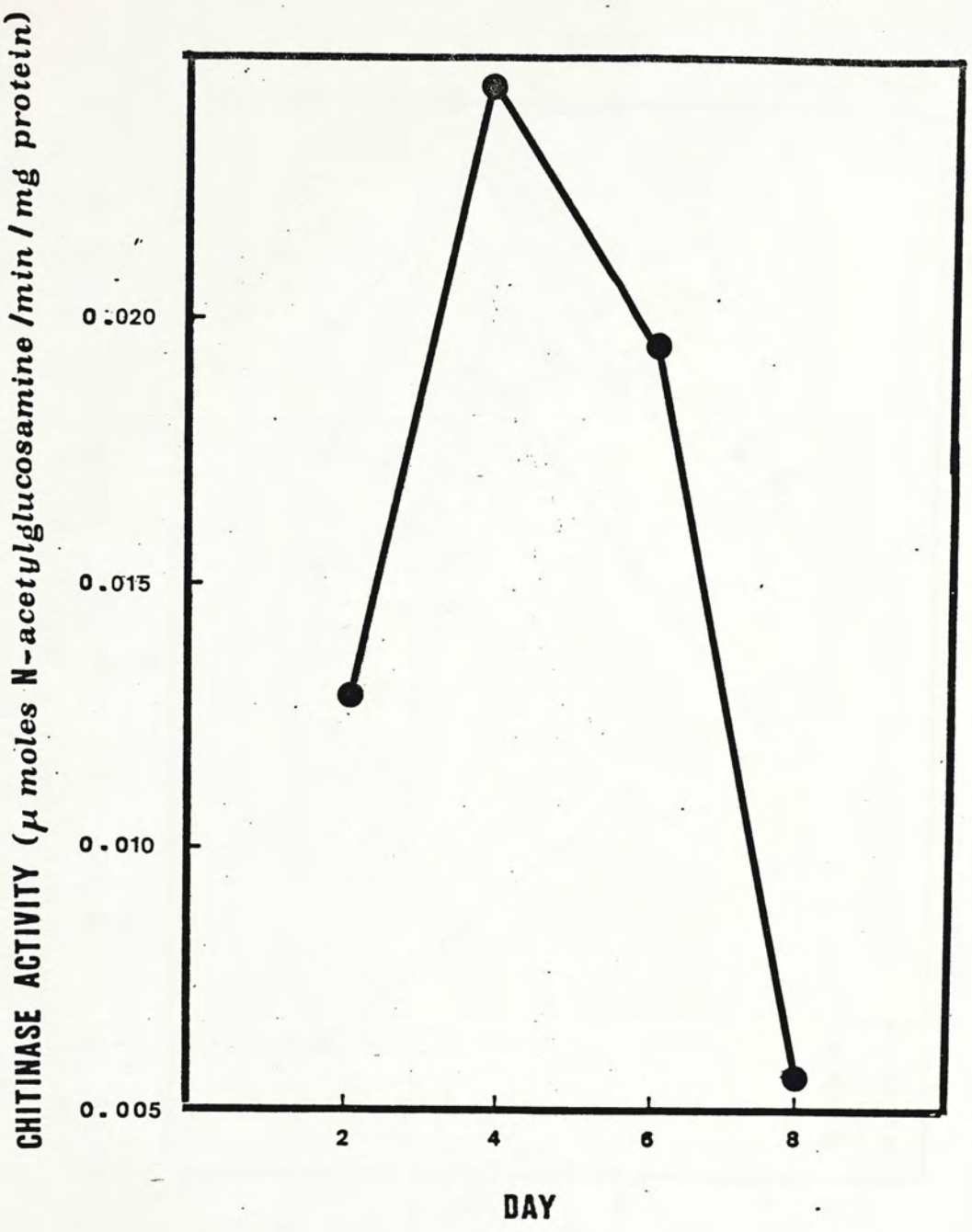


Fig. 3.31. Effect of growth period on chitinase production of T X with inducers of V 5 hyphal wall, Laminaria and chitin

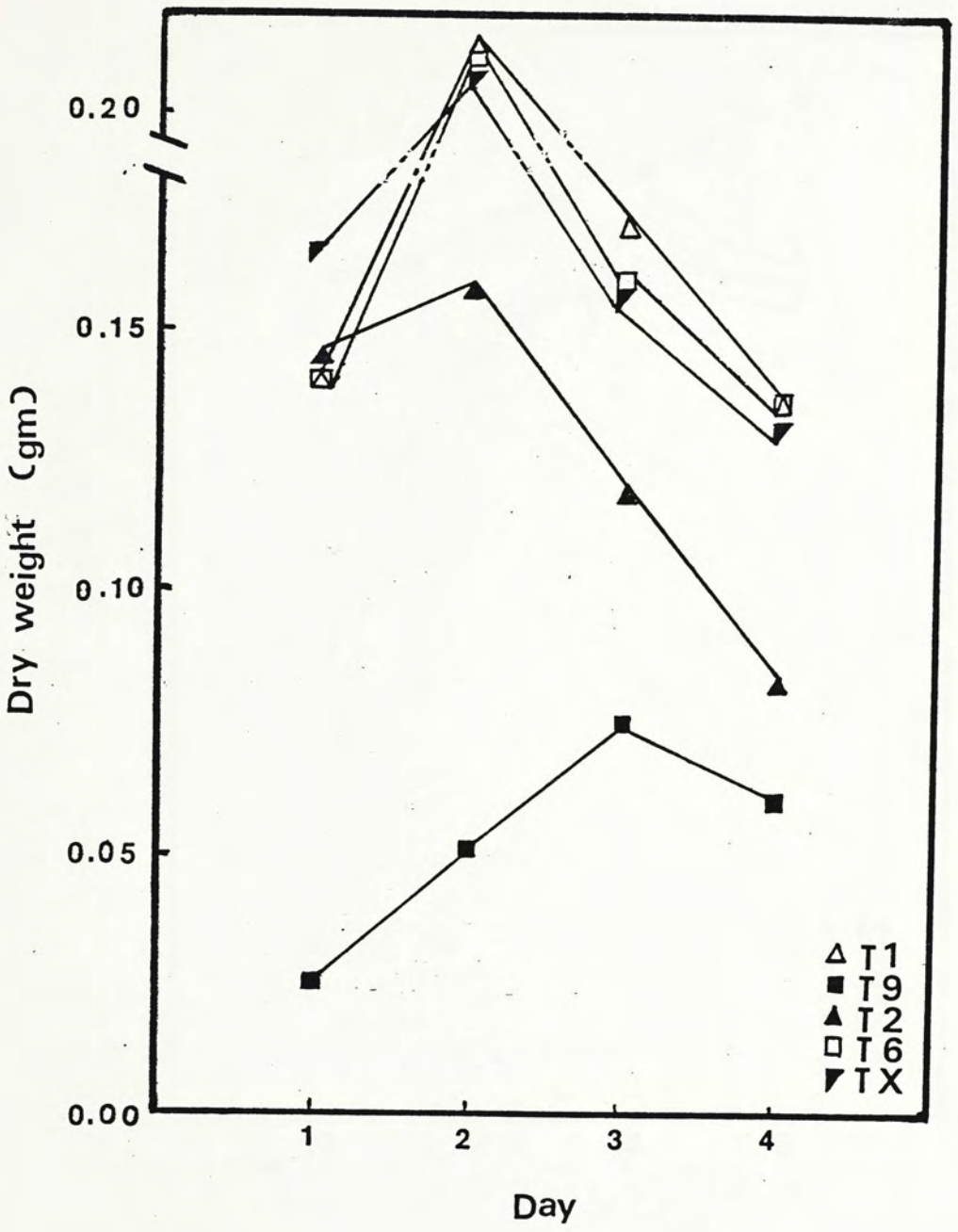


Fig. 3.32. Variation of mycelial dry weight grown in basic TLE medium with incubation time

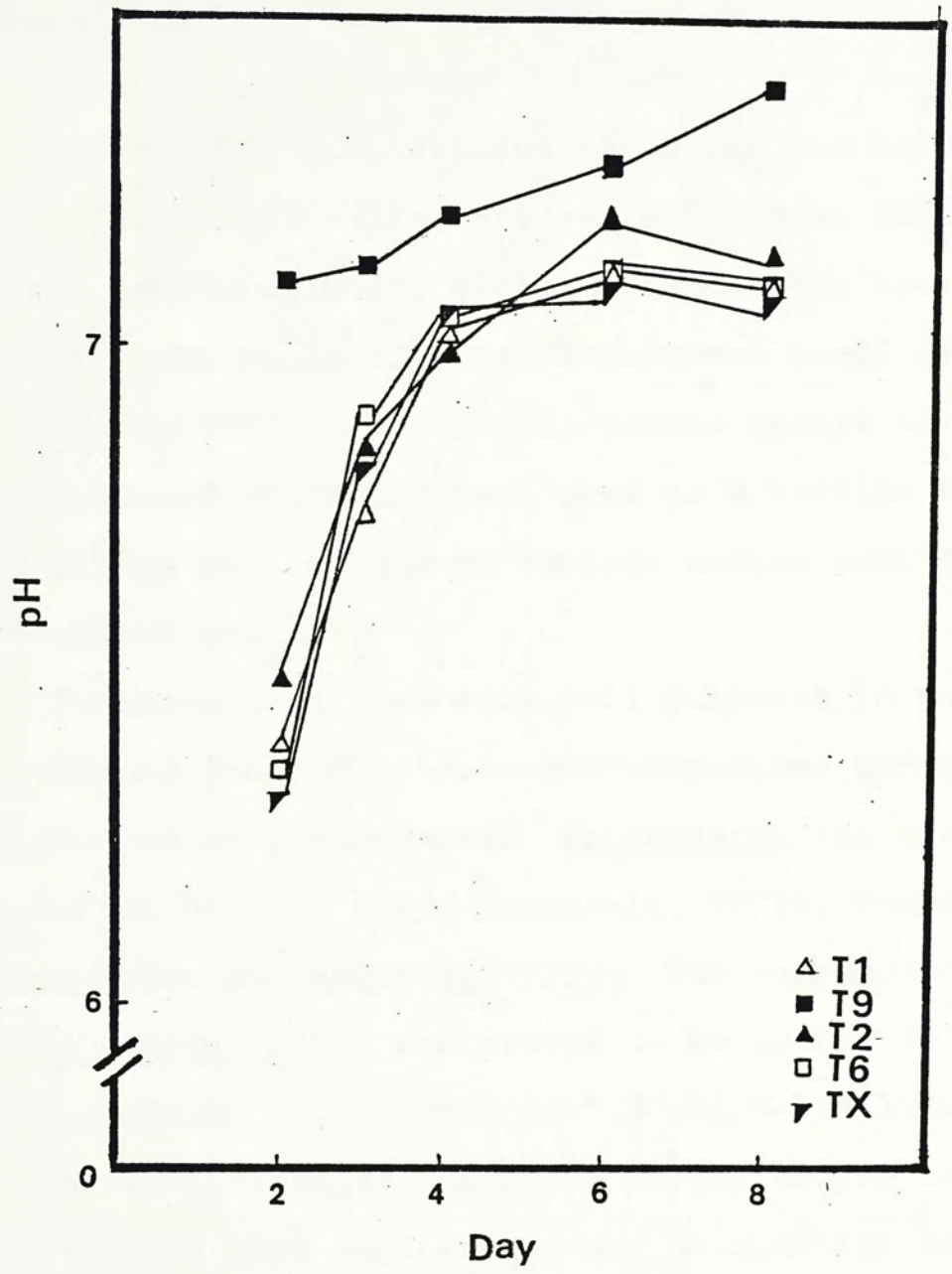


Fig. 3.33. Variation of pH value in basic TLE medium during Trichoderma growth period

3.5 Discussion

Polysaccharide compositions which are the main components of fungal walls, are variable among different fungal species. α-glucan, β-glucan and chitin are the key polymers in cell walls of some filamentous fungi (Farkaś, 1981; Peberdy, 1981). Therefore, enzyme assays of α-, β-glucanases and chitinase were used as a tool to select enzyme samples collected from various medium conditions for protoplast release.

α-1,3-glucan occurs as cell wall polymers in many fungi. It was found that some microorganisms had ability to degrade the α-1,3-linkages. Trichoderma was one of them (Hasegawa and Nordin, 1969; Zonneveld, 1972a; Zonneveld, 1972b; de Vries and Wessels, 1973). The enzyme isolated from Trichoderma viride was proved to be active on both pure α-1,3-glucan and nigeran (α-1,3 and α-1,4-linkages). Thus nigeran was used as substrate in the enzyme assay. It was reported that α-glucanase was an adaptive enzyme in Trichoderma viride. The amount of enzyme activity induced was proportional to the amount of inducer added (Hasegawa and Nordin, 1969). In this experiment, α-glucanases produced by five Trichoderma strains were in agreement with this (Table 3.2 to 3.7). No significant

yield of enzyme was found in control medium of Day 4 culture. In Zonneveld studies (1972a, b), the sudden increase of α-1,3-glucanase after glucose depletion from the medium was conspicuous. Moreover, the enzyme was quite active at pH 8. Thus, maximum enzyme production was obtained in Day 6 culture where the pH of the growth medium was around 8. α-glucanases from Trichoderma in this experiment were similar. The maximum dry weight of Trichoderma mycelia was attained in Day 2 culture on basic TLE medium (Fig. 3.32), and then it dropped down. It might indicated that glucose in the medium was exhausted. It was also shown that pH of the medium increased during growth period (Fig. 3.33). Therefore Day 6 to Day 8 cultures generally allowed for maximum enzyme accumulation in the culture filtrate.

Unlike α-glucanase, a high level of β-glucanase and chitinase in Day 4 culture on basic TLE medium by some Trichoderma strains were detected. In β-glucanase assay, laminarin was used as the substrate. Laminarin is a polysaccharide that can be regarded as a mannitol-containing β-D-glucan in which the main polymeric linkage is β-1,3-linkage. β-D-1,3-glucanase was suggested as an enzyme which can hydrolysis laminarin (Nelson et al., 1963; Bamforth, 1980). The enzyme was previously named

laminarinase by other workers. It was showed that the hydrolysis of laminarin by any fungus was brought about by a group of enzymes, the laminarinase complex, which may comprise a β-glucosidase of low specificity, an exo-β-D-1,3-glucanase and one or more endo-β-D-1,3-glucanase (Chesters and Bull, 1963a, b). In the present study the term β-glucanase was reserved to describe the enzymes. It was previously accepted that β-1,3-glucanase was constitutive in microorganisms (Reese et al., 1961; 1962). However, Bamforth (1980) suggested that exo-β-1,3-glucanase in Trichoderma reesei was inducible and synthesized in response to the presence of food source containing β-1,3-bonds. Chitin, the β-1,4-polymers of N-actylglucosamine was used as the substrate for chitinase assay. In some studies, chitinase was inducible as suitable substance was added (Skujins et al., 1965; Monreal and Reese, 1969). In Jeuniaux's description (1966), chitinase might be synthesized in the absence of inducer (constitutive enzyme) or in its presence (adaptive enzyme). In this experiment, β-glucanase and chitinase were suggested as constitutive enzymes produced by T1 (Trichoderma harzianum) and T2 (Trichoderma koningii). Production of the above mentioned enyzmes by T9 (Trichoderma harzianum), T6 (T. longibrachiatum) and TX were quite low as compared

to T1 and T2 (Tables 3.8 to 3.19). It probably had some variations in enzyme formation among different Trichoderma strains. As in comparison with the effect of growth period to α-glucanase and the above mentioned enzymes, the later reached their maximum yields early than α-glucanase. Changes in pH value in the medium during the growth period might be a factor. Optimum pH of β-glucanases in some organisms was below 6 and enzyme activity dropped down under high pH level (Skujins et al., 1965; Sova et al., 1970). In Chesters and Bull's study (1962c), Trichoderma viride was one of the fungi investigated. It stated that exo-β-1,3-glucanase exhibited optimum activity at pH 4.9 - 5.0, whereas endo-enzymes were most active at pH 6.0. In the study of chitinase, optimum pH was around 5.0 (Tracey, 1955; Jeuniaux, 1966). Thus, it was not suitable for β-glucanase and chitinase in older culture medium where pH value increased.

As previously reported, α-glucanase was an inducible enzyme. In this experiment, the yield of the enzyme in Day 4 culture was low when compared to the yields of other two enzymes. When Laminaria meal was added to the media, enzyme activity increased slightly. In Sternberg and Mandels' study (1979), o-β-D-glucopyranosyl-D-glucose was the most active inducer of cellulase of Trichoderma

reesei. Bamforth (1980) found increased activity of laminarinase when Trichoderma reesei was grown on carboxymethyl-cellulose. It might suggest that enzyme specific to a glucosidic-linkage type are inducible by compounds containing other glucosidic-linkage (Bamforth, 1980). α-glucanase in Trichoderma may be induced by Laminaria meal in this way.

It was found that V5 hyphal wall was an inducer to β-glucanase produced by T1 and T9, two strains of Trichoderma harzianum. Chitin plus V5 hyphal wall was effective in β-glucanase production by T2 (Trichoderma koningii) and T6 (T. longibrachiatum). The positive effect of certain inducers combined was also reported by Laborda et al. (1974). The maximum level for β-1,3-glucanase produced by Streptomyces FL 44 was obtained on Day 2 in chitin-laminarin liquid medium. The response of inducer to β-glucanase production by these microorganisms can not be explained at the present time. Except TX, the medium conditions suitable to maximum production of α-glucanase by the fungi was not the same as to β-glucanase. Laminaria meal had an inducing effect on β-glucanase formation by TX.

Chitin is one of the main components in filamentous fungi. As V5 hyphal wall added to the medium, T1 and T9 (Trichoderma harzianum) were stimulated to produce chitinase.

Chitin supplemented to the medium had a slight effect. However, V5 hyphal wall was not effective in three other Trichoderma species. It was also reported by Monreal and Reese (1969) that little chitinase was produced by Serratia marcesens when the organism was grown with mushroom chitin. In their experiment, it was suggested that the most likely inducer being soluble oligosaccharide portion of the substrate polymer capable of entering the fungal cell. Thus, chitin from V5 hyphal wall might not be the best inducer employed. When it was combined with chitin from crab shells (Sigma), some unknown responses enhanced its inducing effect.

Although this study was done in the presence of α-, β-glucanases and chitinase activities of the enzyme samples, the occurrence of other enzymatic activities might be possible. The absolute quantities of the enzymes vary with the enzyme-producing capacity of different Trichoderma strains. Beside the inducer combinations, variation in the fungus itself, such as autolysis and spore formation of the fungus could also be associated with the enzymes formation (Smith, 1978).

From this enzyme assay, eleven enzyme samples were selected from 70 original samples for protoplast release.

4 PROTOPLAST FORMATION

4.1 Introduction

The straw mushroom, Volvariella volvacea (Bull. ex Fr.) Sing. is a saprophytic fungus of tropics and sub-tropics and is grown quite extensively in the south eastern provinces of China during summer months (Chang, 1972; 1983). In recent years, popularity of this mushroom has become world wide attracting European countries and the United states as well (Santiago, 1982a). Since mid 1960s, much of mycological and agricultural knowledge about Volvariella volvacea have been done by Chang and his co-workers (Li, 1982). In Santiago's study protoplast fusion was used as a tool leading to a new approach to genetic modification of the fungus. The development of fusion technology would be useful to improve strain quality of this fungus and lead to the discovery of high yield strains (Santiago, 1982a). It was found that crude enzyme produced by Trichoderma harzianum was useful in Volvariella volvacea protoplast release (Santiago, 1982a). Qiu et al. (1982) found that Trichoderma longibrachiatum was an effective species in producing lytic enzyme. In this experiment, enzyme

samples from different Trichoderma strains were collected with various inducer combinations. Some relations between enzyme components and fungal wall structures of protoplast formed were discussed. Pleurotus sajor-caju (Fr.) Sing., an edible mushroom, was another fungus used to produce protoplasts by enzyme samples.

4.2 Materials and methods

4.2.1 Materials

Two stains of Volvariella volvacea, V4 and V5, and a strain of Pleurotus sajor-caju PL27 were obtained from Prof. S. T. Chang. They were maintained in complete medium agar plate at 28°C.

4.2.2 Methods

4.2.2.1 Shaking culture method

For protoplast production, mycelia of V4, V5 and PL27 were prepared from cultures grown in liquid complete medium at 28°C. Cultures were incubated in a rotary shaker (100 rotation per min). Mycelia of V4 and V5 cultured for 36 h were collected by filtration, while culture of PL27 was harvested 42 h after incubation. Mycelia of V4 and V5 were suspended in enzyme solution of a final concentration of 20 mg fresh weight per ml. About 40 mg fresh weight of PL27 mycelium was used in one ml enzyme solution.

Suitable amount of freeze-dry enzyme proteins were

dissolved in distill water. Enzyme solutions were mixed with stabilizer solution to a final concentration of 0.6 M stabilizer just before incubated with mycelia. Stabilizer was dissolved in 0.02 M phosphate buffer. Magnesium sulphate was used as stabilizer in enzyme solution when V4 or V5 mycelium was treated. Mycelium of PL27 was resuspended in enzyme solution with 0.6 M KCl. The final pH value was 5.8. The enzyme-stabilizer mixture with mycelium was incubated at 28°C for 3 h on a rotary shaker (100 rotation per min). Protoplast yields were courted by haemocytometer under phase-contrast microscope.

4.2.2.2 Membrane culture method

Mycelium of Volvariella volvacea or Pleurotus sajor-caju was subcultured to solidified complete medium in petri dishes. Sterilized water-permeable cellophanes (dialyzer membrane) with 6 mm in diameter were laid down around margin of the colony on the following day. Cultures were incubated at 28°C. Mycelia grew just over the membrane after one to two days. Membrane was mounted on slide, and then enzyme solution mixed with a suitable stabilizer was added to cover the membrane.

It was incubated at 28°C on shaker and observed under phase-contract microscope.

4.2.2.3 Bursting test

For protoplast production, shaking culture method shown in 4.2.2.1 was used. The enzyme-stabilizer mixture with mycelia was incubated at 28°C for 3 h. The mixture was added on a slide and covered by a coverslide. A drop of distill water was added near the margin of coverslide. Excess water was removed by filter paper at the opposite side of coverslide. Bursting of protoplast was observed under phase-contrast microscope.

4.3 Results

Eleven high enzyme samples were selected from 70 original enzyme samples. In each Trichoderma strain, eight samples were collected in Day 4 culture with different inducer combinations in medium. After enzyme assays, samples with highest amount of enzyme (α-glucanase, β-glucanase or chitinase) were chosen, and then suitable growth period for maximum enzyme yield was tested. The eleven samples were listed in Table 4.1.

Hyphal wall of V5 (Volvariella volvacea) was used as an inducer. When V5 mycelium was mixed with enzyme solution, no protoplast could be found after 3 h. During first thirty minutes of incubation with membrane culture method, it was found that protoplasts bursted rapidly as soon as they were released from the hyphae. The cytoplasm was discharged uncontrollably through a small opening and often extruded as a narrow twisting mass (Figs. 4.1 and 4.2). It was difficult to find a suitable condition even though a wide range of stabilizer (0.3 M to 0.8 M) and enzyme (0.25 to 8 mg protein per ml) concentrations were used.

Another strain of Volvariella volvacea, V4, was also used. It was found that only five enzyme samples

had effect on proloplast release (Table 4.1). The best sample was from Day 4 culture of T6 (Trichoderma longibrachiatum) grown in medium supplemented with V5 hyphal wall and chitin. It contained high yields of chitinase and β-glucanase. The four other enzyme samples contained large amount of either chitinase or β-glucanase. It appeared that chitinase and β-glucanase were the essential components. When enzyme samples had small amount of either enzyme, there was no protoplast release.

Higher yields of protoplasts could be prepared from PL27 (Pleurotus sajor-caju) as compared to those from V4. Cell wall of PL27 seemed to be more sensitive to enzyme samples than V4, except with enzymes collected on Day 4 culture produced by T2 (Trichoderma koningii) in the presence of V5 hyphal wall and chitin. Enzyme samples produced by T2 were not effective to release protoplasts from PL27.

Protoplasts from the same fungus generally were in similar size even though enzyme samples were different. Protoplasts of Volvariella volvacea and Pleurotus sajor-caju with different sizes were observed after 3 h incubation (Fig. 4.3 A, B). Vacuoles could be seen in V4 protoplasts. Protoplasts from PL27 were small spherical bodies and non-vacuolated. On occasions when very effective

enzyme samples were used to treat PL27 mycelium, it was found that all the hyphal walls appeared to be digested, leaving a mass of protoplasts loosely bound to each other (Fig. 4.3 C).

Colonies of PL27 usually grew rapidly and became compactly tufted on membrane culture, so that it was difficult to observe the process of protoplast release. On the other hand, it was clearer to observe the process of protoplast release from Volvariella volvacea (Fig. 4.4). Protoplasts could be released from hyphal tips or older hyphal compartments. They appeared to be released through pores in hyphal walls.

As protoplasts from V4 and PL27 were exposured to distill water, there were extensive bursting of the protoplasts. After addition of distill water, protoplasts near the margin of coverslide bursted quickly for the hypotonic condition. Observation of protoplasts near the centre of the coverslide was found a short delay before protoplasts began to burst; they swelled slightly and bursted suddenly. Vacuoles presented in protoplasts were remained in sight for few minutes.

Table 4.1. Production of protoplasts from Volvariella volvacea V4 and Pleurotus sajor-caju PL27 by Trichoderma enzymes

Enzyme Sample* (Trichoderma species: Inducers**: growth period)	Enzyme activity			Protoplast yield X 10^6/ml	
	α-glucanase (μ mole glucose/ min/mg protein)	β-glucanase (μ mole glucose/ min/mg protein)	Chitinase (μ mole N-acetyl-glucosamine/ min/mg protein)	V4	PL27
T1: V+L: 8 days	0.061	0.331	0.041	none	3.04
T1: V: 4 days	0.008	0.675	0.117	0.04	27.7
T9: V+L+C: 6 days	0.141	0.529	0.012	none	0.07
T9: V: 4 days	0.022	0.785	0.198	0.32	30.4
T2: L+C: 6 days	0.055	0.361	0.109	none	none
T2: V+C: 4 days	0.015	0.769	0.107	0.16	none
T2: L+C: 4 days	0.051	0.447	0.124	none	none
T6: V+L+C: 6 days	0.052	0.291	0.021	none	none
T6: V+C: 4 days	0.015	0.598	0.556	1.28	31.16
TX: L+C: 8 days	0.181	0.179	0.037	none	0.78
TX: V+L+C: 4 days	0.07	0.473	0.025	0.04	2.84

* Enzyme concentration: 2 mg protein/ml

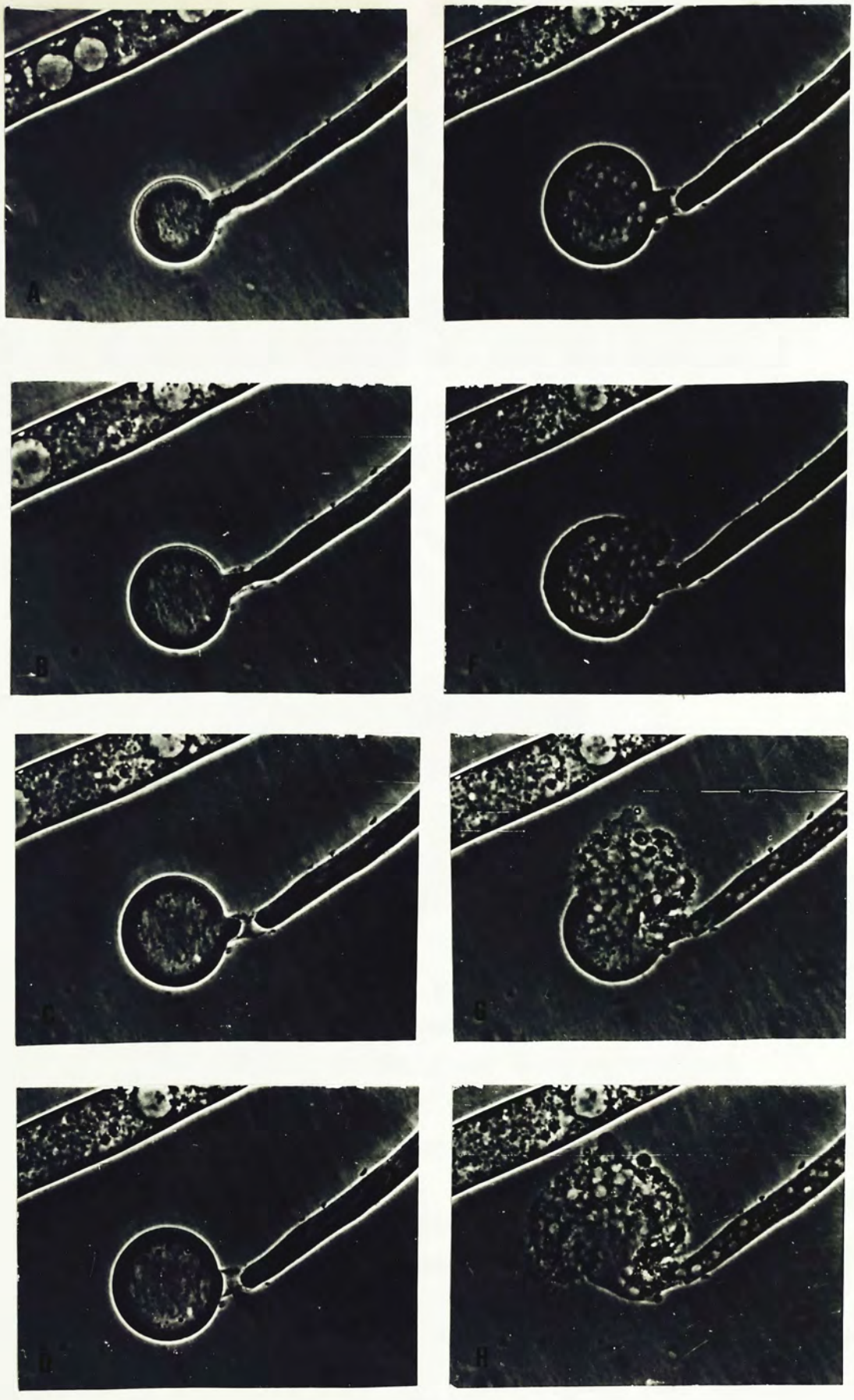
** V: V5 hyphal wall; L: Laminaria meal; C: Chitin

Fig. 4.1. Bursting of protoplast from V5 mycelium incubated in 0.6 M $MgSO_4$ stabilized enzyme mixture at 28°C. X 1200.

A-D: Protoplast forming from tip of mycelium.

E-H: Bursting appeared within hyphae.

Arrow pointing bursting site.


A
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G

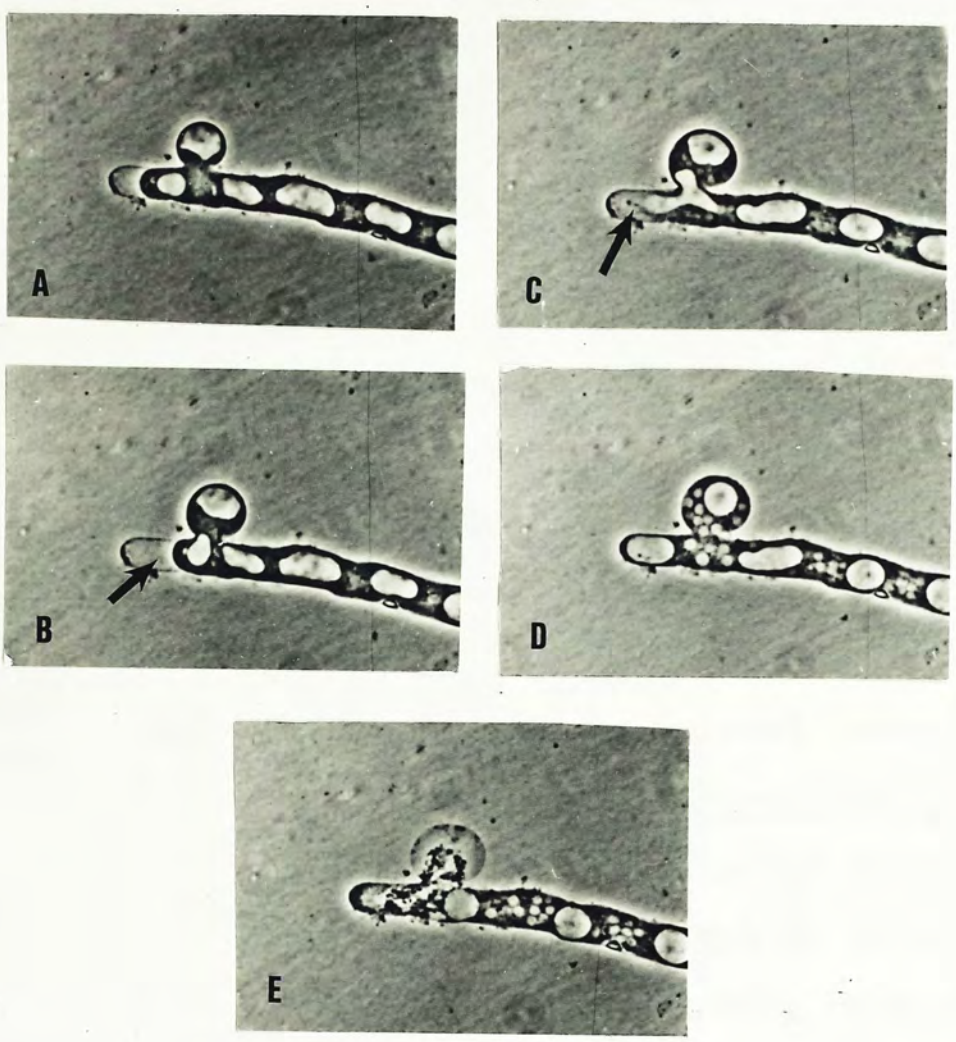


Fig. 4.2. Bursting of protoplast from V5 mycelium incubated in 0.6 M $MgSO_4$ stabilized enzyme mixture at 28°C. X 1200.

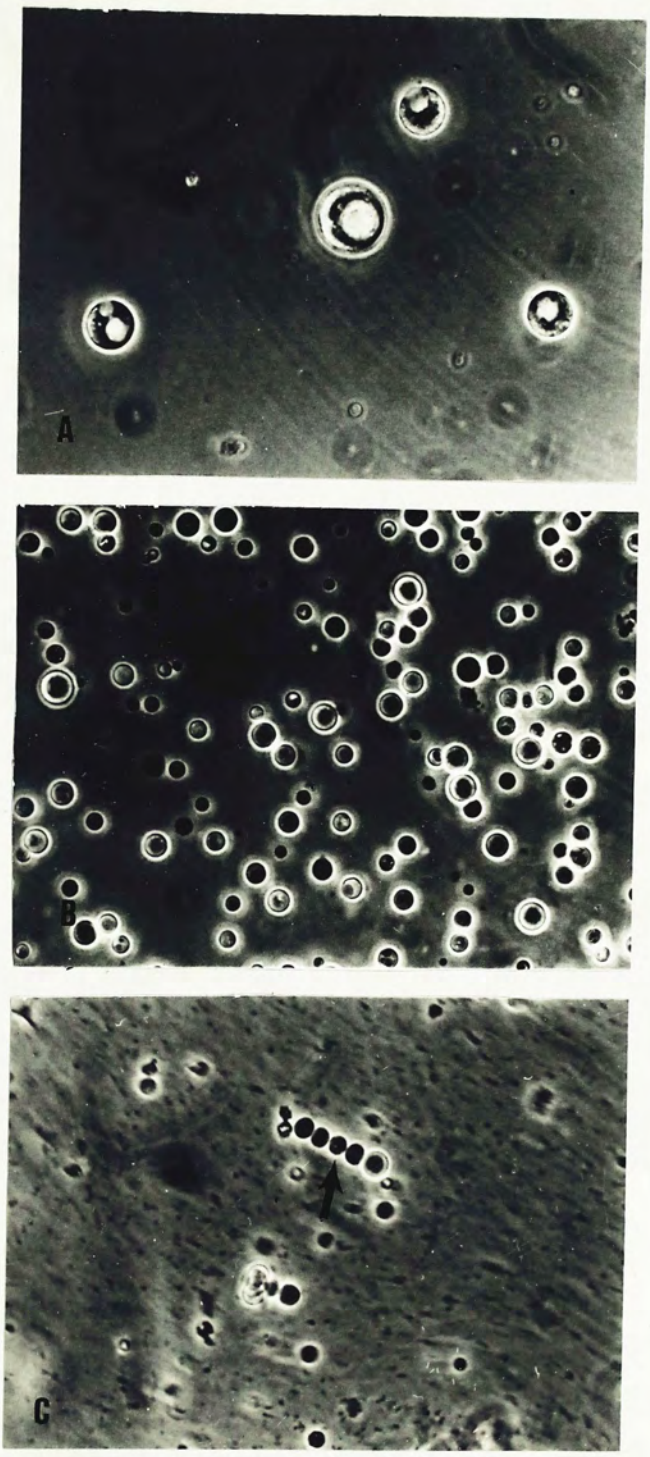
A-B: Cytoplasm passing through opening of cell wall to form protoplast.

C-E; Bursting appeared within hyphae and cytoplasm returning back to empty hyphal tip.

Arrow pointing bursting site.

Fig. 4.3.A: Larger and vacuolated protoplasts released from Volvariella volvacea incubated in 0.6 M $MgSO_4$ stabilized lytic mixture at 28°C after 3 h. X 1200.

B-C: Smaller and non-vacuolated protoplasts released from Pleurotus sajor-caju incubated in 0.6 M KCl stabilized lytic mixture at 28°C after 3 h. Arrow pointing mass of protoplasts loosely bound to each other. X 1200.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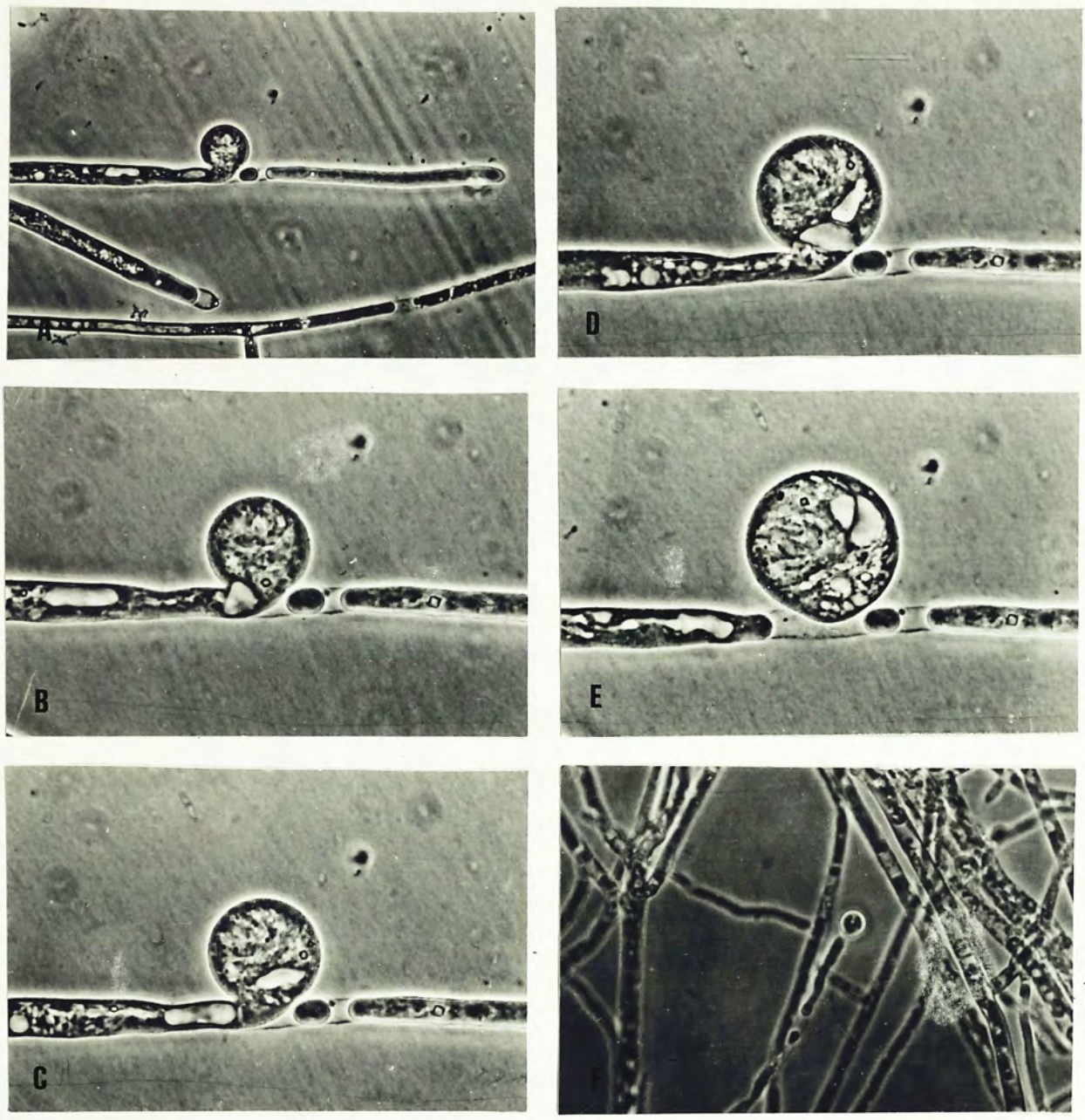

Fig. 4.4.A-E: Protoplast released from older hyphal compartment of Volvariella volvacea incubated in 0.6 M $MgSO_4$ stabilized enzyme mixture at 28°C. A: X 600. B-E: X 1200.

F: Protoplast released from hyphal tip. X 600.

4.4 Discussion

Hyphal wall of V5, a Volvariella volvacea strain, was used as an inducer. When it was used as material for protoplast formation, no protoplast was found even though treated with a commerical enzyme, Novozyme 234. When V5 was cultured in liquid complete medium, normal appearances were observed as compared to other Volvariella volvacea strains, e.g. V4. However, it had a small, slow-growing mat of mycelia with little aerial hyphae on solid complete medium plate. It was named "atypical type". Cells of atypical hyphae had a smaller nuclear number (Chang and Yau, 1971). Probably it had some weakness in hyphal wall or membrane structure, so that releasing protoplasts were easier by bursting when hypha of V5 was treated with lytic enzymes.

It was stated by Hamlyn et al. (1981) that with the filamentous fungi protoplasts were readily detected microscopically as osmotically sensitive spherical bodies. Bursting test in this experiment confirmed that the spherical bodies released from mycelia were protoplasts for their sensitivity to osmotic pressure.

In general, high levels of ß-glucanase and chitinase activities were associated with their enzyme complexes

that gave best protoplast yield (Hamlyn et al., 1981). Similar results were obtained in this experiment when V4 was used. In Hamlyn et al. studies, only relatively small numbers of protoplasts from Volvariella volvacea were produced by commerical enzymes as compared to other fungi tested. Similarly, low yields of protoplasts were produced from V4 as compared to PL27 in this experiment. It was found that percentage of chitin present in hyphal walls of V5 and PL27 were about 38% and 40% respectively in 3 days cultures; and about 53% and 61% respectively of R-glucan (β-glucan) occurred in V5 and PL27 (personal communication with Li, G. S. F., Department of Biology, The Chinese University of Hong Kong, 1983). Chitin and β-glucan are the main components in fungal walls of V5 and PL27, therefore chitinase and β-glucanase are important in protoplast release from the fungi. However, hyphal wall of PL27 contained only small amount (0.88%) of S-glucan (α-glucan) as compared to V5 (13.9%) (personal communication with Li, 1983). Hence, the amount of α-glucanase in enzyme samples seemed to be not related to protoplast yield from PL27. In de Vries and Wessels' studies (1973), it was observed that no protoplast was released from Volvariella volvacea when Helicase, an enzyme lacking α-1,3-glucanase, was used. In this experiment, if enzyme

sample contained a low level of α-glucanase activities, then small numbers of protoplasts were produced from V4 with the enzyme. Moreover, it was observed that three enzyme samples from T2 (Trichoderma koningii) had no effect on protoplast release from PL27. Not only the presence of β-glucanase and chitinase but also the occurrence of other enzymes in Trichoderma enzyme samples are related to the protoplast release. Enzyme samples from T2 might lack some essential components to induce protoplast formation.

In this experiment, the effective enzyme sample was produced by T6 (Trichoderma longibrachiatum) in medium supplemented with V5 hyphal wall and chitin. Novozym 234 (Novo), a suitable commercial enzyme for stimulating protoplast formation by several fungi (Hamlyn et al., 1981), was compared for its enzyme activities against the best enzyme sample from T6 in this experiment. It was found that α-glucanase, β-glucanase and chitinase activities of Novozym 234 were 0.056 μ mole glucose per min per mg protein enzyme, 0.571 μ mole glucose per min per mg protein enzyme and 0.021 μ mole N-acetylglucosamine per min per mg protein enzyme respectively. Thus, the best enzyme sample had a similar level of β-glucanase activity as compared to Novozym 234, and more than twenty times

higher of chitinase activity than this commercial enzyme. However, α-glucanase activity of the enzyme sample was only 0.015 μ mole glucose per min per mg protein enzyme. Thus, V5 hyphal wall used as inducer did not stimulate high yield of α-glucanase. In de Vries and Wessels' study (1972), cell walls of Schizophyllum commune were effective to induce α-glucanase. In Qiu et al. study (1982), enzyme from Trichoderma longibrachiatum was active to stimulate protoplast formation from Volvariella volvacea. Debris of sugar-cane was the main component in the medium. Fungal wall and debris of sugar-cane were cheap as compared to nigeran which is used as α-glucanase inducer. Probably other materials containing α-glucan could also induce Trichoderma to produce high yield of the enzyme.

This study is in its initial stage to search for a suitable condition in stimulating the production of Trichoderma lytic enzymes. Trichoderma longibrachiatum is an effective species to be used. It is hoped that furth experimentations will lead through improvement of the inducer combinations to stimulating more effective enzymes. In this study, the enzyme samples selected have potential not only stimulate Volvariella volvacea protoplast release, but also have potential to stimulate protoplast production by other filamental fungus.

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